A STUDY ON AEROBIC BACTERIAL AND FUNGAL ISOLATES IN BRONCHOALVEOLAR LAVAGE IN PATIENTS WITH LOWER RESPIRATORY TRACT INFECTION IN A TERTIARY CARE HOSPITAL

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CERTIFICATE

This is to certify that this dissertation titled **"A STUDY ON AEROBIC BACTERIAL AND FUNGAL ISOLATES IN BRONCHOALVEOLAR LAVAGE IN PATIENTS WITH LOWER RESPIRATORY TRACT INFECTION IN A TERTIARY CARE HOSPITAL"** is a bonafide record of work done by **DR. S. HEMALATHA,** during the period of her Post graduate study from June 2008 to April 2011 under guidance and supervision in the Institute of Microbiology, Madras Medical College and Government General Hospital, Chennai-600003, in partial fulfillment of the requirement for **M.D. MICROBIOLOGY** degree Examination of The Tamilnadu Dr. M.G.R. Medical University to be held in April 2011.

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DECLARATION

I declare that the dissertation entitled **"A STUDY ON AEROBIC BACTERIAL AND FUNGAL ISOLATES IN BRONCHOALVEOLAR LAVAGE IN PATIENTS WITH LOWER RESPIRATORY TRACT INFECTION IN A TERTIARY CARE HOSPITAL "** *submitted by me for the degree of M.D. is the record work carried out by me during the period of* ***May 2009 to May 2010*** *under the guidance of Professor* ***Dr. S. GEETHALAKSHMI M.D., Ph.D.,*** *Professor of Microbiology, Institute of Microbiology, Madras Medical College, Chennai. This dissertation is submitted to the Tamilnadu Dr.M.G.R. Medical University, Chennai, in partial fulfillment of the University regulations for the award of degree of M.D., Microbiology (Branch IV) examination to be held in April 2011.*

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CONTENTS

INTRODUCTION

Respiratory infections may be upper or lower respiratory tract infections. Lower respiratory tract infections include pneumonia, lung abscess, bronchitis, bronchiolitis etc.

Lower respiratory tract infections are the major cause of morbidity and mortality worldwide. They remain the leading cause of deaths among all infectious diseases and they account for 3.9 million deaths worldwide and 6.9% of all deaths.[105]

Lower respiratory tract infections are particularly more common in immunocompromised patients like HIV patients, transplant recipients, patients with neoplasms etc.

Although rapid determination of the etiologic agents is of paramount importance in managing respiratory infections, the responsible pathogens are not determined in as many as 50% of patients despite extensive diagnostic tests.[6] The specimens used for the diagnosis of lower respiratory tract infections can be sputum, endotracheal aspirate, transtracheal aspirations, bronchoscopy specimens, lung puncture or biopsy.

Examination of sputum has been the primary means of diagnosis but the drawback is the difficulty of some patients to mobilize the lower respiratory secretions and the possible contamination of sputum with oropharyngeal flora.[101] Other procedures like transtracheal aspirate, lung

puncture or lung biopsy are highly invasive and can cause pneumothorax or bleeding.

Bronchoscopic specimens include bronchial washings or aspirate, bronchoalveolar lavage, protected bronchial brush samples. Fiberoptic bronchoscopy is the technique frequently used for obtaining these respiratory specimens.

Bronchial washings or aspirates can also be contaminated with upper respiratory tract flora but more relevant than sputa.

BAL is a deeper sampling of desquamated host cells and secretions and now reported to have considerable value in diagnosing pulmonary infections. The value of this technique in conjunction with quantitative cultures for the diagnosis of most of the major respiratory tract pathogens, including bacterial pneumonia, has been documented.[25,73] BAL is especially suitable for detecting cysts of Pneumocystis jirovecii, fungal elements and mycobacteria.[23,96] BAL has been shown to be a safe and practical method for diagnosing opportunistic pulmonary infections in immunocompromised patients.

Bronchoalveolar lavage involves the injection of 30 to 50 ml of physiological saline through a fiberoptic bronchoscope and using the aspirate for smear preparation and culture.

Direct microscopic evaluation of smears provide immediate information about the causative organism and is helpful in starting antimicrobial therapy, but culture of the microbial pathogens is considered to be the gold standard. Semi-quantitative or quantitative cultures of respiratory secretions obtained by alveolar lavage techniques have been recommended for the diagnosis of pneumonias particularly in intubated patients undergoing ventilation.[26]

Antimicrobial susceptibility tests are mandatory to monitor the efficiency of available antimicrobial agents and the emergence of drug resistance among bacterial and fungal isolates.

Considering the importance of bronchoalveolar lavage in the diagnosis of lower respiratory tract infections, the present study was conducted to identify the common aerobic bacterial and fungal isolates and their antimicrobial susceptibility profile in patients with respiratory infections attending a tertiary care hospital in Chennai.

AIM OF THE STUDY

- To isolate and identify the common aerobic bacterial agents in bronchoalveolar lavage
- To isolate the common fungal agents in bronchoalveolar lavage
- To evaluate the sensitivity and resistance pattern of all the bacterial agents isolated
- To study the sensitivity and resistance of the fungal isolates to the commonly used antifungal drugs

REVIEW OF LITERATURE

Lower respiratory tract is the part of the respiratory tract below the vocal cords. While often used as a synonym for pneumonia, the rubric of lower respiratory tract infection can also be applied to other types of infection including lung abscess, bronchiolitis, bronchitis etc.

Bronchitis

Bronchitis can be classified as either acute or chronic. Most often acute bronchitis is caused by viral infection and hence antibiotic therapy is not indicated in immunocompetent individuals.[114,111] A small proportion of cases of acute bronchitis have a non viral cause. However, in the group with the more prolonged course, termed infectious bronchitis, *Mycoplasma pneumoniae* and *Bordetella pertussis* play a more important role. In adolescents and adults with prolonged cough, *Bordetella.pertussis* has been associated with 12% to 32% cases.[29] *Chlamydia pneumoniae* strain TWAR respiratory tract infections have also included cases with the clinical features of acute bronchitis.[60,59]

Chronic bronchitis is symptomatically similar to acute bronchitis, but more prolonged and less intense. Bacteria predominate, particularly *Streptococcus pneumoniae*, non-capsulated *Haemophilus influenzae* and *Moraxella catarrhalis.*[77]

Bronchiolitis

Bronchiolitis is an acute lower respiratory tract illness that occurs during the first 2 years of life. The syndrome is caused primarily by viral infections. The characteristic clinical manifestations include an acute onset of wheezing and hyperinflation, most commonly associated with cough, rhinorrhea, tachypnea and respiratory distress.[82]

Respiratory syncitial virus is clearly the major pathogen and the para influenza viruses are the second most commonly isolated agent with *Parainfluenza type 3* predominantly.[67] Occasionally *Mycoplasma pneumoniae* may cause bronchiolitis.

Pneumonia

The symptoms of pneumonia were described by Hippocrates (c. 460 BC – 370 BC). Hippocrates referred to pneumonia as a disease "named by the ancients." Maimonides (1138–1204 AD) observed "The basic symptoms which occur in pneumonia and which are never lacking are as follows: acute fever, sticking [pleuritic] pain in the side, short rapid breaths, serrated pulse and cough."

Bacteria were first seen in the airways of individuals who died from pneumonia by Edwin Klebs in 1875. Initial work identifying the two common bacterial causes *"Streptococcus pneumoniae"* and *"Klebsiella pneumoniae"* was performed by Carl Friedlander and Albert Frankel in 1882

and 1884, respectively. Friedlander's initial work introduced the Gram stain, a fundamental laboratory test still used to identify and categorize bacteria. Christian Gram's paper describing the procedure in 1884 helped differentiate the two different bacteria and showed that pneumonia could be caused by more than one microorganism.

Sir William Osler, known as "the father of modern medicine," appreciated the morbidity and mortality of pneumonia, describing it as the "captain of the men of death" in 1918, as it had overtaken tuberculosis as one of the leading causes of death in his time. However, several key developments in the 1900s improved the outcome for those with pneumonia. With the advent of penicillin and other antibiotics, modern surgical techniques, and intensive care in the twentieth century, mortality from pneumonia dropped precipitously in the developed world. Vaccination of infants against *"Haemophilus influenzae"* type b began in 1988 and led to a dramatic decline in cases shortly thereafter. Vaccination against *"Streptococcus pneumoniae"* in adults began in 1977 and in children began in 2000, resulting in a similar decline.

In the past, pneumonia was typically classified as community-acquired, hospital-acquired, or ventilator-associated. Over the last decade or two, patients presenting to the hospital have often been found to be infected with multidrug-resistant (MDR) pathogens previously associated with

hospital-acquired pneumonia. Factors responsible for this phenomenon include the development and widespread use of potent oral antibiotics, earlier transfer of patients out of acute-care hospitals to their homes or various lower-acuity facilities, increased use of outpatient IV antibiotic therapy, general aging of the population, and more extensive immunomodulatory therapies.

The potential involvement of these MDR pathogens has led to a revised classification system in which infection is categorized as either community-acquired pneumonia (CAP) or health care–associated pneumonia (HCAP), with subcategories of HCAP including hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP).[66]

The aetiology of pneumonia varies according to whether it has been acquired in the community or in hospital and the risk factor present. Many of the bacteria found as colonizers of the upper respiratory tract have been implicated in pneumonia. Antibiotic treatment and hospitalization affect the colonizing flora, leading to an increase in number of aerobic gram negative bacilli.[92]

These factors affect the sensitivity and specificity of sputum culture as a diagnostic test and results must always be interpreted in the light of clinical information.[101] Sputum culture results are often unreliable and sensitivity of culture is poor for many pathogens, although culture and antibiotic

sensitivity may be of value in sputum specimen from patients with severe exacerbation of COPD.[62]

1. Community Acquired Pneumonia:

The commonest cause of CAP is *Streptococcus pneumoniae*, which is responsible for up to 60% of cases in community based surveys and may be multi-drug resistant. It can affect individuals of any age, including those without known risk factors. Other bacterial pathogens tend to cause pneumonia in the presence of specific risk factors. Patients with COPD are additionally at risk of pneumonia caused by *Haemophilus influenzae*, and *Moraxella catarrhalis* as are patients infected with HIV. *Staphylococcus aureus* pneumonia occurs either in the context of recent influenza infection or, less commonly as a result of blood borne spread from a distant focus, COPD or aspiration. Aerobic Gram-negative rods are rare causes of community acquired pneumonia. Occasionally, *Klebsiella pneumoniae*, can cause pneumonia typically in patients with a history of alcohol abuse and homelessness (Friedlander's pneumoniae).

Mycoplasma pneumoniae cause up to 20% of community acquired pneumonia, second only to *Streptococcus pneumoniae*. It tends to occur in epidemics every 4 to 5 yrs and affects younger age groups. *Chlamydia pneumoniae* is an exclusively human pathogen[53], but pneumonia caused by *Chlamydia psittaci* and *Coxiella burnetii* occurs in individuals with the

relevant exposure history (bird and farm animals). These agents are responsible for a minority of cases. *Legionella pneumophila* is a rare cause of outbreaks of community acquired pneumonia.

2. Hospital acquired pneumonia:

Hospital acquired pneumonia is the second commonest type of nosocomial infection. Risk is increased by the presence of underlying diseases and by various interventions and procedures. Mechanical ventilation is a major risk factor. Patients with critical illnesses requiring prolonged ventilation are susceptible to multi-resistant *Pseudomonas aeruginosa* and *Acinetobacter species* (eg. *Acinetobacter baumanii*). Aerobic Gram-negative bacilli, including members of the Enterobacteriaceae (such as *Klebsiella* and *Enterobacter sp*.) and *Pseudomonas aeruginosa* are implicated in up to 60% cases.[45]

Lung abscess

Lung abscess results when microbial infection causes necrosis of the lung parenchyma producing one or more cavities. These cavities often communicate with large airways resulting in cough with purulent sputum. Although many organisms may produce lung abscess, most cases are due to anaerobic mouth flora and follow aspiration.

The predominant organisms responsible for lung abscess are bacteria, specifically mouth anaerobes that are normal flora in the gingival crevices.[11] In the presence of periodontal disease, the gingival crevice deepens and fills with anaerobic gram-negative organisms.[110] Studies employing sample collection techniques that avoid contamination with oral flora combined with good anaerobic culture methods showed that anaerobes are found in about 90% of lung abscesses and are the only organisms present in about half of cases.[9] The most frequently isolated anaerobes are *Peptostreptococcus* spp., *Fusobacterium nucleatum,* and *Prevotella melaninogenica*.[83,40]

Monomicrobial lung abscess occasionally may be caused by bacteria including *Staphylococcus aureus,* enteric gram-negative rods such as *Klebsiella* spp., *Pseudomonas aeruginosa, Burkholderia pseudomallei* (melioidosis), *Pasteurella multocida, group A streptococcus*, *Haemophilus influenzae* types b and c, *Legionella* spp., *Rhodococcus equi, Actinomyces* spp., and *Nocardia* spp. *Streptococcus pneumoniae,* particularly type 3, has been reported to cause lung abscess, but cavitation in the setting of pneumococcal pneumonia may be due to concomitant infection with anaerobes.[81] Other organisms that can cause lung abscess include many fungi, mycobacterial species, and parasites (e.g., *Paragonimus westermani, Entamoeba histolytica*).

Oropharyngeal colonization with *P. aeruginosa,* other aerobic gram-negative rods, and, less often, *S. aureus* is a common event in hospitalized patients, particularly patients who receive ventilatory support.

Common Aetiological agents of Lower Respiratory Tract Infections

A) BACTERIA	**B) FUNGI**	**D) ATYPICAL**
1)Streptococcus pneumoniae	1) Aspergillus spp	1) Mycoplasma pneumoniae
2)Staphylococcus aureus	2) Candida spp	2) Chlamydophila psittaci
3)Haemophilus influenzae	3) Pneumocystis jirovecii	3) Chlamydophila pneumoniae
4)Enterobacteriaciae	4) Histoplasma capsulatum	4) Legionella spp
a) Escherichia coli	5) Cryptococcus neoformans	**E) RICKETTSIAL**
b) Klebsiella spp	6) Coccidioides imitis	1) Coxiella burnetti
c) Enterobacter spp	**C) VIRAL**	2) Rickettsia rickettsiae
d) Serratia spp	1) Children	**F) MYCOBACTERIAL**
e) Pseudomonas aeruginosa	a)Respiratory syncitial virus	1)Mycobacterium tuberculosis
5)Mixed anaerobic bacteria	b)Para influenza viruses	2) Non tuberculous mycobacteria
a) Bacterioides spp	c) Influenza A virus	**G) PARASITIC**
b) Fusobacterium spp	2) Adults	1) Ascaris lumbricoides
c) Peptostreptococcus spp	a) Influenza A virus	2) Strongyloides stercoralis
d) Prevotella spp.	b) Influenza B viru	3) Toxoplasma gondii
e) Peptococcus spp	c) Adeno virus types 4&7	4) Paragonimus westermanii

Fungal infections:

Invasive pulmonary aspergillosis is an increasingly common problem in hospitalized patients receiving corticosteroids, especially in patients who are immunocompromised and those with prior pulmonary disease. *Aspergillus fumigatus* is the commonest *Aspergillus spp.* to infect humans.[43] The use of galactomannan testing of serum and BAL increases the diagnostic yields, as do research studies using molecular detection methods.[44] Pulmonary manifestations in hosts without major impairment of cellular or humoral immunity include allergic bronchopulmonary aspergillosis in patients with asthma or cystic fibrosis, chronic cavitary pulmonary aspergillosis and single aspergilloma.

Pneumocystis jirovecii pneumonia is a frequent opportunistic infection among AIDS patients. HIV infected patients are at high risk of developing Pneumocystis pneumonia when the CD4+ count is below 250/mm^{3}.[94] Diagnostic confirmation of PCP is achieved by microscopic detection of *Pneumocystis jirovecii* in respiratory specimens taken by expectoration, induced sputum, open-lung biopsy, transbronchial biopsy, bronchoscopy or bronchoalveolar lavage.

Some unusual fungal causes of lower respiratory tract infections are endemic to defined geographical areas. The diagnosis should be considered in travelers returning from endemic areas who present with respiratory

illness or pneumonia, particularly if they fail to respond to standard therapy. These infections include histoplasmosis caused by *Histoplasma capsulatum,* coccidioidomycosis caused by *Coccidioidis immitis* and *Coccidioidis pedrosii* and blastomycosis caused by *Blastomyces dermatidis*. Although these infections have distinguishing characteristics, it is often difficult to differentiate them clinically from other causes of respiratory infection, particularly in their early stages. Paracoccidioidomycosis caused by *Paracoccidioides braziliensis* usually causes asymptomatic primary pulmonary infection that may reactivate if immune function decline.

Cryptococcus neoformans is an unusual cause of pneumonia, usually in normal hosts and may be associated with meningitis. It has a world wide distribution.

Candida spp. are rare causes of lower respiratory tract infections. Occasionally infections occur as a result of hematogenous seeding. Diagnosis is difficult given that the airways may become colonized in compromised hosts treated with antibiotics.

Mycobacterium tuberculosis:

Mycobacterium tuberculosis was discovered by Robert Koch in 1882. The 1990 World Health Organization report on the Global Burden of Disease ranked tuberculosis as the seventh most morbidity-causing disease in the world and expected to continue in the same position up to 2020.[87]

The initial diagnostic approach to suspected cases of pulmonary tuberculosis is to demonstrate mycobacterium tuberculosis in stained smears of expectorated sputum. In most of the tuberculosis centers, even after meticulous search, the bacteriological positive yield from sputum is around 16-50% large portion remain negative in spite of clinical profile and radiological lesions being consistent with diagnosis of pulmonary tuberculosis.[79] The transmission rate of sputum smear negative tuberculosis as compared to smear positive tuberculosis is reported as 22%.[42] It has been reported that 74% of them develop active tuberculosis in five years, if not treated.[68]

Culture of sputum for Acid Fast Bacilli takes long time and a reliable serological test is not yet available. In such a situation bronchoscopy has been tried for rapid diagnosis of tuberculosis in smear negative cases.

Nocardia and Actinomyces infections[4,10,14]

Nocardiosis and Actinomycosis are rare conditions that may affect other systems apart from the lungs.

Nocardia spp. are most often seen in the lungs and cause necrotizing pneumonia. This is commonly associated with cavitation. It may also produce a slowly enlarging pulmonary nodule and pneumonia that is often associated with empyema. *Actinomycetes spp.* cause a thoracic infection that

may involve the lungs, pleura, mediastinum or chest wall. Aspiration of oral contents is a risk factor for the development of thoracic actinomycosis.

Parasitic infections:

Several helminthic infections may give rise to the syndrome Tropical Pulmonary Eosinophilia, characterized by patchy pulmonary infiltrates and eosinophilia accompanied by symptoms of cough, fever and weight loss. These signs and symptoms are associated with passage of larval forms through the lungs and include *Ascaris lumbricoides*, *Ankylostoma duodenale* and *Strongyloides stercoralis*. The lung fluke, *Paragonimus westermanii* has a wide distribution and is particularly prevalent in the Far East, India and West Africa. Human infection is acquired by consumption of uncooked freshwater crabs or crayfish that harbor encysted metacercariae. Ova of *Paragonimus westermanii* are demonstrable in sputum.[86]

LAB DIAGNOSIS OF LOWER RESPIRATORY INFECTION

Sputum examination:

Microscopic examination and culture of expectorated sputum remain the mainstay of the laboratory evaluation of pneumonia despite ongoing controversy concerning their sensitivity and specificity.[116] However, lower respiratory tract secretions will be contaminated with the upper respiratory secretions especially saliva and will be contaminated with normal oropharyngeal flora as it passes through the mouth.[6,77] Some individuals

like HIV patients and children have difficulty in mobilizing the lower respiratory secretions.

Endotracheal aspirate:

The lower respiratory tract may be sampled by introducing a catheter through the larynx into the trachea. Endotracheal aspirates can also be contaminated because oral secretions can dribble down the pathway of the endotracheal tube. This technique has not been widely adopted nowadays.[77]

Transtracheal aspirates:

Transtracheal aspirates are obtained by inserting a small plastic catheter into the trachea via a needle previously inserted though the skin and cricothyroid membrane. This procedure has reduced chance of contamination with upper respiratory flora. But due to the invasive nature and too frequent complications, this technique is rarely used.

Bronchoscopic techniques for the diagnosis of LRTI:

The protected specimen brush [PSB] and Bronchoalveolar lavage (BAL) are the two techniques that have evolved for the diagnosis of LRTI. Several investigators believe that bronchoscopic sampling is the gold standard of care in the diagnosis and management of VAP.[27]

Protected specimen brush technique:

This technique was initially described by Wimberley et al, who using an in vitro model of upper airway colonization showed that protection

against contamination was provided by a sampling device protected by a double sheathed plugged catheter . The methodology was later standardized by Meduri et al.[84]

Bronchoalveolar Lavage:

In this technique the bronchoscope is wedged in the airway of the lung to be sampled, and the lavage fluid is introduced and aspirated in a standardized manner.[84] BAL samples approximately 10 million alveoli and thus represents a larger area than PSB.

In patients who are unable to raise sputum spontaneously and in whom attempts to induce sputum production are unsuccessful, Fibreoptic bronchoscopy is usually the initial procedure. Analysis of bronchoalveolar lavage fluid may increase the diagnostic yield of bronchoscopy, especially in immunocompromised persons such as patients with AIDS and suspected opportunistic infections or patients with suspected non infectious causes of chronic pneumonia.[38,95]

Non Bronchoscopic distal airways sampling:

The endotracheal tube bypasses the proximal airways and allows catheters to be passed blindly to sample secretions from distal airways. The potential advantages of nonbronchoscopic sampling techniques are less invasiveness, less cost, absence of contamination by the bronchoscopic channel, and less procedure- related risks to the patient. However, sampling

errors can be inherent in a blind technique where the airways are not visualized.

The nonbronchoscopic techniques include :

1. Plugged telescoping catheter (PTC),

2.Protected bronchoalveolar mini-lavage (mini-PBAL), and

3."Blind" PSB.

The overall concordance between the bronchoscopic and nonbronchoscopic techniques is around 80% which would mean the diagnosis could be missed by nonbronchoscopic techniques especially when the pneumonia affects the upper lobes of the left lung.[72] Therefore the bronchoscopic methods are perhaps the preferred technique whenever the option is available and the patient's condition is stable.

Lung puncture and biopsy:

Percutaneous aspiration or needle biopsy may be performed blindly or under fluoroscopic guidance, particularly if a localized lesion is present.[117] Open lung biopsy is the most invasive procedure and reserved for situations in which other measures failed.[77]

BRONCHOALVEOLAR LAVAGE

For more than 20 years, lavage of the bronchial tree through a rigid bronchoscope has been used in the management of severe asthma[115] and of alveolar proteinosis.[100] Once Myrvik et a1[90] had shown that in rabbits

lavage could yield alveolar macrophages, the technique formed the basis of the new topic of pulmonary cell biology, now rapidly developing and advancing our understanding of a wide variety of pulmonary diseases. The methods used for sampling the cells of the pulmonary inflammatory and immune systems of man have progressed through lavage via the rigid bronchoscope[76] or via a large balloon-tipped catheter wedged into a primary or secondary branch of the bronchial tree[54,65] to the present-day bronchoalveolar lavage via the fibreoptic bronchoscope.

Since its introduction by Professor Shigeto Ikeda in 1964, the use of flexible fiberoptic bronchoscopy (FOB) is expanding and it is considered to be one the most important breakthrough in diagnostic pulmonology.

Bronchoalveolar lavage (BAL) is a specialised technique, described by Reynolds and Newball[103] in 1974, which consists instillation of small quantity of saline directly into distal airways and recovering the aspirate through FOB. This procedure is repeated until a total of 100- 300 ml of saline has been instilled. Usually between 40% and 70% of the infused volume is recovered, but in patients with destructive lung disease and airflow obstruction the proportion is smaller; recovery correlates inversely with the severity of the airflow obstruction.[61]

Analysis of postmortem lung biopsy has shown that BAL fluid cultures to be as useful as PSB cultures. When the results of 11 studies

evaluating BAL fluids from a total of 435 ICU patients with suspected VAP were pooled, the overall accuracy of this technique was similar to that of PSB.[41] Therefore, it is not necessary to perform both BAL and PSB in the same patient.[97] BAL is easier to apply, less expensive and does not require the specialized brush. It also allows a larger area of the lung to be sampled.

A study by Barreiro et al, showed the sensitivity and specificity of BAL fluid. Culture of Bronchoalveolar lavage fluid had sensitivity of 87%, specificity 91%, positive predictive value of 87% and negative predictive value 91%.[7] In a study by Castella et al, sensitivity was 82% and specificity was 44%. In another study by Guler et al, the sensitivity was 83% and positive predictive value was 50%.[63]

BAL is considered as the clinical sample of choice for the investigation of *Pneumocystis jirovecii.*[93] Stains such as Gomori's methenamine silver and Toluidine blue O for cysts and Giemsa for trophozoites of *Pneumocystis jirovecii,* can be performed on smears of respiratory specimens[39,64]. The Toluidene blue and Giemsa staining of bronchoalveolar lavage fluid is more sensitive and specific for the detection of *Pneumocystis jirovecii.*[78]

Analysis of BAL for Acid Fast Bacilli including culture for *Mycobacterium tuberculosis* has significant role to establish the diagnosis

when extensive search for AFB in expectorated sputum has repeatedly failed, when sputum expectoration is absent or sputum induction has failed.

Caminero et al, concluded that bronchoscopy should be conducted on all patients without expectoration and negative sputum smear and that BAL performance should be a routine procedure as it is simple and usually uncomplicated technique.[24] Among various bronchospic specimen BAL is considered the best for the diagnosis of tuberculosis.[119] BAL has significant sensitivity and specificity in a study by Conde et al, and was useful in the diagnosis of PTB in 72% cases.[36]

Advantages of BAL[15]

1) This technique is safe and less invasive.
2) It samples a large area of lung parenchyma.
3) The recovery of large volume of secretion makes BAL suitable for a series of microscopic analysis that enable early identification of patients with pneumonia.
4) BAL fluid can be cultured using quantitative technique.
5) Contamination with upper respiratory flora is prevented.
6) More useful in isolating *Pneumocystis jerovicii* from immunocompromised patients.
7) More sensitive and specific than the sputum sample for the isolation of *Mycobacterium tuberculosis.*

Complications of bronchoalveolar lavage are rare. Transient respiratory distress and syncope have been reported,[35] but these are also seen in patients undergoing fibreoptic bronchoscopy without bronchoalveolar lavage. The most frequent complication is fever, but even this is seen in fewer than 3% of patients and rapidly responds to antibiotic treatment.

Procedure:

As the patient arrives in the bronchoscopy suite (or if the patient is already in the hospital), an intravenous catheter (IV) will be started for administration of medication and IV fluids. The patient is then connected to a monitor for continuous monitoring of the heart rate, blood pressure, and oxygen level in the blood. If needed, supplemental oxygen will be supplied either through a cannula or a facemask.

Patients will be lying on their back with oxygen supplemented through the mouth or the nose. Prior to the insertion of the flexible bronchoscope, a local anesthesia with topical lidocaine is given in the nose and to the back of the throat. The flexible bronchoscope can be introduced either through the mouth or the nose. Some patients may require an endotracheal tube to be inserted through the mouth, passing the vocal cord, and into the trachea to protect and secure the airway. Once the bronchoscope is in the airway, an additional topical anesthetic will be sprayed into the airway for local

anesthesia to minimize discomfort and coughing spells. A total of 100-300 ml of 0.9% physiological saline in instilled through the bronchoscope. The saline is then aspirated in three aliquots as first, second and third sample for microbiological processing. A minimum of 20 ml is necessary for the processing.

Diagnostic techniques:

a) Microscopic examination of smears

b) Culture of bacterial and fungal isolates.

The sample received is centrifuged at 3000 rpm for 15 mts. The supernatant is discarded and the deposit is used for processing.[12]

A. Microscopic evaluation of smears:

a) 10% KOH Mount

A drop of BAL is placed over a clean glass slide, a drop of 10% potassium hydroxide reagent is added over the specimen and a coverslip is placed over it taking care to avoid trapping of air bubbles. Proteinaceous components, such as host cells are partially digested by the alkali, leaving the intact polysaccharide containing fungal cell walls.[74] Tube KOH can be used for extended clearing of cells. Clearing can be enhanced by gentle heating.

In 1998, Sharma et al have reported that KOH preparations demonstrated fungus in 100 percent of total culture proven cases.[108]

Bharathi et al in 2006 have concluded that the direct microscopic examination of KOH mount is a rapid, reliable and inexpensive diagnostic modality, which would facilitate the institution of early antifungal therapy before culture results become available.[17] In 1993 Vajpayee et al reported that 10% KOH mounts demonstrated fungus in 94.3% of total culture proven cases.[118]

b) Gram's stain

Gram's staining of bronchoalveolar lavage enables to look for Gram positive or Gram negative organisms and helps in early diagnosis. In fungal infections, it helps to identify the gram positive yeast cells with hyphae or pseudohyphae. For bacteria, Gram's staining is the most frequently used procedure and provide morphological information that can be used in the empirical selection of antibiotics for therapy.[13] Results of quantitative culture are not available until 24-72 hours after the procedure and potentially contribute to the frequent rate of morbidity and mortality.[113]

Sole Violan et al found a correlation between Gram's staining and BAL culture results.[112] In a study by Frederic Duflo et al, the presence of micro organism in the initial Gram's stain is totally correlated with positive quantitative cultures. The sensitivity of Gram's staining was 76.2%, specificity 100%, positive predictive value 100% and negative predictive value 75.4%.[56]

c) Ziehl Neelsen stain

Ziehl Neelsen stain (Acid-fast stain) is a useful differential staining procedure that specifically stains all members of the genera mycobacteria. Being unassociated with the human flora, finding of acid-fast bacilli in bronchoalveolar lavage is strongly indicative of an active infection with *Mycobacterium tuberculosis*.

d) Modified Ziehl Neelsen stain

It is called modified Kinyon's acid fast staining used for the identification of other Acid fast organisms like *Nocardia, Cryptosporidia* etc. The concentration of sulphuric acid used is 1%. The filaments of *Nocardia spp.* are partially acid fast when stained by modified acid fast technique.

e) Toluidene blue O stain

Toluidene blue O stain is used for staining the cysts of *Pneumocystis jirovecii*. It is especially useful in immunocompromised patients like patients with HIV infection. Toluidene blue staining of bronchoalveolar lavage fluid has increased sensitivity and specificity for the identification of cysts of *Pneumocystis jirovecii*.[58]

f) Giemsa stain

Giemsa staining has been routinely used for the detection of trophozoites and intracystic bodies in smears of bronchoalveolar lavage fluid

(BAL) from patients with *Pneumocystis jirovecii* pneumonia. Sulphation of smears before staining with Giemsa allows cysts to be visualised, thus enabling a single stain to be used to show all the stages of *Pneumocystis jirovecii* in BAL or sputum, which is particularly useful, considering the increase in the prevalence of *Pneumocystis jirovecii* pneumonia in conjunction with the spread of AIDS.[120]

Bacterial culture:

Semi-quantitative loop method is used for culturing bacteria. A calibrated loop is used to plate out 0.01ml of fluid. The deposit of centrifuged sample is spreaded on MacConkey agar, Chocolate agar and Blood agar plates by standard method and incubated at 37ºC for 24 hrs. Sample with colony count of $\geq 10^4$ cfu/ml is considered to have significant growth for Bronchoalveolar lavage fluid.[18]

Fungal culture:

The sample is centrifuged and the deposit is inoculated onto two Sabourauds Dextrose Agar slants and incubated at 25º and 37ºC.

Interpretation of growth:

Bacterial:

The specific identification of bacterial pathogens was based on the microscopic morphology, staining characteristics and biochemical properties using standard laboratory criteria.[1]

Fungal:

Fungal isolates were identified by studying the colony morphology, colony colour, production and arrangement of conidia in preparations stained by Lactophenol cotton blue stain.[49] When identification is difficult due to inadequate sporulation, Riddle's slide culture technique can be employed.[47]

Antimicrobial Susceptibility Testing:

As resistance patterns to commonly used antibiotics and antifungals for the treatment of lower respiratory tract infections continue to shift, sensitivity testing play an important role in appropriate management of individual cases based on susceptibility characteristics and for community surveillance.

Antibacterial Susceptibility:

Antibacterial sensitivity testing was performed by the Kirby-Bauer disc diffusion technique, using 0.5 McFarland's turbidity as the standard inoculum density on Mueller Hinton agar plates. Commercially available Hi-Media antibiotic discs were used.

Antifungal Susceptibility:

The recent increased incidence of fungal infections and the growing number of new antifungal agents have multiplied the demand and interest for in vitro antifungal susceptibility testing.[1]

Antifungal susceptibility testing can be performed by[88]

Agar based methods:

1. Agar dilution method

2. Disc diffusion method

3. E-test method

Broth based methods:

1. Broth macrodilution method

2. Broth microdilution method

3. Calorimetric methods.

MATERIALS AND METHODS

PERIOD OF STUDY

This is a cross sectional study undertaken over a period of one year from May 2009 to May 2010.

PLACE OF STUDY

This study was carried out at the Institute of Microbiology, Madras Medical College, Chennai and Institute of Thoracic Medicine, Government General Hospital, Chennai.

STUDY MATERIAL

All bronchoalveolar lavage samples from patients with lower respiratory tract infections collected by fibreoptic bronchoscopy in the Institute of Thoracic Medicine, Government General Hospital, Chennai

ETHICAL CONSIDERATIONS

Written consent to participate in the study was obtained from the patients or their guardians after providing full explanation of the study. This study was reviewed and approved by Institutional Ethical Committee, Madras Medical College & Government General Hospital, Chennai 3. All data were handled confidentially and anonymously.

STATISTICAL ANALYSIS

Statistical analyses were carried out using Statistical Package for Social Sciences (SPSS) and Epi-Info softwares by a statistician. The

proportional data of this cross sectional study were tested using Pearson's Chi Square analysis test and Binomial proportion test.

SPECIMEN COLLECTION

Fibreoptic bronchoscopy is inserted transnasally or per orally, minimum 60 ml of 0.9% physiological normal saline is instilled and then aspirated. A minimum of 20 ml should be collected for processing in the microbiology laboratory.

SPECIMEN PROCESSING

Three sets of samples were taken, centrifuged at 3000rpm for 15 mts and the deposit was processed as follows

1. First set of sample was used for microscopic examination.
    a) 10% Potassium hydroxide (KOH) mount preparation,
    b) Gram's stain procedure
    c) Ziehl-Neelsen staining
    d) Modified Ziehl-Neelsen staining,
    e) Toluidine blue O staining
    f) Giemsa staining.
2. Second set of sample was inoculated onto solid media like blood agar, chocolate agar and MacConkey agar.
3. Third set of sample was inoculated onto two slants of Sabouraud's dextrose agar (SDA) devoid of antibiotics and cycloheximide.

a) Microscopic Examination:

a) 10% KOH mount preparation was examined for the presence of septate or aseptate hyphal elements, conidial forms or yeast forms.

b)Gram positive or Gram negative organisms or yeast cells were looked for in Gram's stain preparation.

c) Fixed smear was stained by acid fast staining and looked for any acid fast bacilli.

d) Modified acid fast staining was done for identifying *Nocardia spp*.

e) Smear was stained with Toluidene blue and Giemsa stains and examined for the presence of trophozoites and cysts of *Pneumocystis jirovecii.*

b) Culture:

The centrifuged deposit of bronchoalveolar lavage was inoculated on MacConkey agar, blood agar and chocolate agar plates and incubated at 37ºC for 24 hrs. The two inoculated SDA slants were incubated at 25ºC and 37ºC for up to 4 weeks.[101]

Interpretation of Bacterial culture:

Bacterial culture plates were observed for growth at 24 hours. If there are < 10 colonies on the plate, that equates < 10^3 cfu/ml, between 10-100 colonies indicate 10^3-10^4 cfu/ml and 100-1000 colonies indicate 10^4-10^5

cfu/ml. The diagnostic threshold for bronchoalveolar lavage is $\geq 10^4$cfu/ml.[12]

Bacterial isolates were identified by means of Gram's staining, motility and biochemical reactions by standard microbiological techniques as recommended by Clinical and Laboratory Standards Institute (CLSI).

All bacterial isolates were preserved in 0.2 – 0.5 % semisolid Nutrient agar slopes at 4 – 5º C in refrigerator with periodic subculture every 2 months.[104]

Interpretation of Fungal culture:

Two SDA slants were inoculated and incubated at 25ºC and 37ºC for minimum of 4 weeks before reporting as negative. These slants were inspected daily during the first week and twice weekly during the next three weeks for growth.[70] Growth on the slants with presence of hyphal elements in 10% KOH preparation was regarded as significant fungal growth.

Identification of filamentous fungi was done by preparing Lacto Phenol Cotton Blue mount and studying the morphology of hyphae and conidial arrangement. In difficult, ambiguous cases where sporulation was inadequate, Riddle's slide culture technique was performed.[47]

Lactophenol Cotton Blue stain:[69]

Place a drop of Lactophenol Cotton Blue Stain in the center of a clean slide. Remove a fragment of the fungus colony 2-3mm from the colony edge

using an inoculating or teasing needle. Place the fragment in the drop of stain and tease gently. Apply a coverslip. Do not push down or tap the cover slip as this may dislodge the conidia from the conidiophores. Examine the preparation under low and high, dry magnification for the presence of characteristic mycelia and fruiting structures.

Riddle's slide culture technique:[47]

This was used to study the undisturbed morphological details of fungi, particularly the relationship between reproductive structures and mycelium.

Procedure:

1. A round piece of filter paper was placed on the bottom of a sterile Petri dish. A pair of thin glass rods was placed on top of the filter paper to serve as supports for a 3 inch × 1 inch glass microscopic slide.
2. A small 1cm block of SDA previously poured into a Petridish was placed on the surface of the microscopic slide. The block was cut using a sterile scalpel.
3. A small portion of the fungal colony to be studied was inoculated onto three or four places in the margins of the agar block using a straight inoculating wire.
4. A coverslip was gently heated by passing it quickly through the flame of a Bunsen burner and immediately placed directly on the surface of the inoculated agar block.

5. A small amount of water was placed into the bottom of the petri dish to saturate the filter paper.
6. The Petridish was incubated at 30ºC for 3-5 days.
7. When a growth visually appeared to mature, the coverslip was gently lifted from the surface of the agar with a pair of forceps taking care not to disrupt the mycelium adhering to the bottom of the coverslip.
8. The coverslip was placed on a small drop of LPCB on a second glass slide. Likewise, the mycelium adhering to the surface of the original glass slide after the block was removed also was stained with LPCB and a fresh coverslip was overlaid.
9. The characteristic shape and arrangement of spores was observed microscopically.

In case of yeasts, identification and speciation was done by Gram's stain morphology, germ tube test, morphology on corn meal agar, and biochemical tests by standard microbiological techniques as recommended by CLSI.

Gram's stain

Gram positive oval budding yeasts cells with presence or absence of pseudohyphae.

Germ Tube test

A loopful of creamy white yeast like colony from SDA was taken and it was inoculated into 0.5ml of mammalian serum. It was incubated at 37ºC for one and a half to 2 hours. After incubation period, a loop full of this serum suspension was placed on a clean glass slide and covered with cover slip and focused under high power objective to see the characteristic germ tube formation .

Chromagar

A single colony from Sabourauds dextrose agar was taken and it was streaked on chromagar and incubated at 37°C for 48 hrs. After incubation period, the plates were observed for characteristic coloured colonies of candida.

Cornmeal agar

A single colony from Sabouraud's dextrose agar was inoculated on to a plate of cornmeal agar containing 1%Tween 80 and trypan blue. Three parallel steaks were made about half an inch apart at a 45º angle to the culture medium. A sterile coverslip was placed over it and incubated at 30ºC for 48 hrs. After incubation the areas where the cuts into the agar were made were examined for the presence of blastoconidia, arthroconidia, pseudohyphae, hyphae or chlamydoconidia.[5]

Carbohydrate fermentation test[5]

A saline suspension of the yeast colonies was prepared. About 0.2ml of this suspension was inoculated on to the carbohydrate fermentation broth that contain Durham's tube and different sugars at a concentration of 2%. The different sugars used were glucose, lactose, sucrose, maltose respectively. The tubes were incubated at room temperature for 7-10 days. After incubation period, the tubes were observed for acid and gas production.

Carbohydrate assimilation test[5]

Organisms from SDA were inoculated onto carbohydrate free medium either in nutrient agar or blood agar. A suspension of the yeast in saline or distilled water to a density equivalent to a McFarland No.4 standard was prepared. The saline suspension was swabbed on sterile
yeast nitrogen base agar plate.

The different carbohydrate disc used include glucose, lactose, sucrose, maltose, galactose, trehalose , raffinose. The disc were placed on to the surface of the agar approximately 30mm apart from each other. The plates were incubated at 30ºC for 24 to 48hrs.

After incubation, the plates were observed for the presence of a colour change around the carbohydrate containing discs or the presence of growth surrounding them. All fungal growths were preserved by suspending a small

inoculum of spores or conidia in sterile distilled water and kept in sterile cryo vials at room temperature.[70]*

SENSITIVITY TESTING OF ISOLATES

ANTIBACTERIAL SUSCEPTIBILITY TESTS:

Bacterial isolates were subjected to antibiotic sensitivity by the Kirby-Bauer's Disc Diffusion technique on Mueller Hinton agar plates as recommended by CLSI. Peptone water culture of the bacterial isolates corresponding to 0.5 McFarland's turbidity was used as inoculum. The entire dried agar surface was evenly streaked in three different directions with a sterile cotton swab dipped into the inoculum.[104]

Commercial Hi-Media Antibiotic discs were used. Maximum six antibiotic discs were used for each 9cm diameter petridish.. These plates were incubated at 37ºC for 16–18 hours in ambient air. The diameters of zones of inhibition were interpreted according to CLSI standards[33] for each organism. Media and discs were tested for quality control using standard strains.

The following standard strains were used

Staphylococcus aureus-ATCC 25

Escherichia coli-ATCC 25922

Pseudomonas aeruginosa-ATCC 27853

The antibiotic discs used for gram negative bacilli were:

Antimicrobial agent	Inhibition zone in mm		
	Resistant	Intermediate	Sensitive
Amikacin 30µg	14	15-16	17
Amoxicillin/clavulanic acid 20/10µg	13	14-17	18
Ceftazidime 30 µg	14	15-17	18
Cefotaxime 30 µg	14	15-22	23
Ciprofloxacin 5 µg	15	16-20	21
Cotrimoxazole 1.25/23.75 µg	10	11-15	16
Gentamicin 10 µg	12	13-14	15
Imipenem 10 µg	13	14-15	16

The antibiotic discs used for Gram Positive Cocci were:

Antimicrobial agent	Inhibition zone in mm		
	Resistant	Intermediate	Sensitive
Amikacin 30 µg	14	15-16	17
Amoxicillin/clavulanic acid 20/10 µg	13	14-17	18
Ampicillin 10 µg	28	-	29
Ceftriaxone 30 µg	13	14-20	21
Cefotaxime 30 µg	14	15-22	23
Ciprofloxacin 5 µg	15	16-20	21
Cotrimoxazole 1.25/23.75 µg	10	11-15	16
Erythromycin 15µg	13	14-22	23
Oxacillin 1µg	10	11-12	13
Vancomycin 30 µg	-	-	15

CLSI 2010

Minimum inhibitory concentration (MIC) for detecting Methicillin resistance:[34]

MIC was performed for Oxacillin by broth microdilution method for *Staphylococcus aureus* isolates to detect Methicillin Resistant *Staphylococus aureus* (MRSA).

The test was performed using Mueller Hinton broth with 2% Sodium chloride in a microtitre plate. The bacterial suspension adjusted to 0.5 McFarlands turbidity was further diluted ten times to give a final concentration of 5×10^5 CFU/ ml in each well.

The range of concentration of Oxacillin used was 0.125µg/ml – 32µg/ml. After inoculation, the microtitre plates were incubated in ambient ir at 35ºC for 24 hours. The drug controls and the growth controls were included in each test.

Interpretation

The MIC value is the lowest concentration of Oxacillin that completely inhibits visible growth of the test organism. Growth in the wells with Oxacillin dilutions should be compared with the growth in the control wells for determining the end point.

Screening of ESBL producing strains:

Clinical and Laboratory Standards Institute (CLSI) has developed screening test for identifying the ESBL producing strains.

According to CLSI guidelines, strains showing zones of inhibition ≥ 27mm for cefotaxime were selected for conformational tests of ESBL. *Klebsiella pneumoniae* ATCC 700603 ESBL positive strain was used as control along with *Escherichia coli* ATCC 25922 as negative control.

ESBL confirmatory test:

1. Double Disc Synergy test (DDST)[71]

The isolated colonies were inoculated in peptone water at 37°C for 2-6 hrs. The turbidity was adjusted to 0.5 Mc Farland's standard and lawn culture was made on Muller Hinton agar using sterile swab. Augmentin disc (20/10 µg) was placed in the centre of plate. Both side of Augmentin disc, a disc of cefotaxime (30 µg) and cetazidime (30 µg) were placed with centre to centre distance of 15mm to centrally placed disc. The plate was incubated at 37°C overnight, ESBL production was interpreted as the 3rd generation Cephalosporin disc inhibition was increased towards the Augmentin disc or if neither discs were inhibitory alone but bacterial growth was inhibited where the two antibiotics were diffused together.

2. Phenotypic Confirmatory Disc Diffusion Test (PCDDT) for ESBL[71]

Antibacterial susceptibility testing was done on Muller Hinton Agar with 0.5 McFarlands standards of the organism. Lawn Culture of the organism was made and 3rd generation Cephalosporins Cefotaxime (30µg) disc and Cefotaxime with Clavulinic acid (30 µg + 10 µg) disc was placed

with 25mm apart. An increase of ≥ 5mm in zone of inhibition for Cefotaxime with Clavulinic acid compared to Cefotaxime was confirmed as ESBL producers.

ANTIFUNGAL SUSCEPTIBILITY TESTS:

The antifungal susceptibility tests was done by two methods

1) DISC DIFFUSION METHOD

2) BROTH MICRODILUTION METHOD

Disc diffusion method

This method was used for the *Candida* species.

Inoculum preparation:

The inoculum suspension was prepared by picking five colonies, each of atleast 1mm in diametrer, from 24 hour old culture of *Candida spp.* and suspending the material in 5ml of sterile saline. The suspension was then adjusted spectrophotometrically at 530nm to match the transmittance produced by 0.5 McFarland's barium sulphate standard. This procedure produces an inoculums size 1×10^6 to 5×10^6cfu/ml. The following standard strain was tested each time to ensure quality control : *Candida albicans* ATCC 90028

Procedure

It was performed on Muller Hinton agar plate supplemented with 2% glucose and 0.5 µg/ml methylene blue.[31]

Antifungal susceptibility testing was carried out following the M44-A, National Committee for Clinical Laboratory Standards (NCCLS) guidelines, using Amphotericin B, Fluconazole and Itraconazole antifungal discs.

The entire dried agar surface was evenly streaked in three different directions with a sterile cotton swab dipped into the inoculum suspension. The plate was allowed to dry for 20 minutes. Using a pair of flame sterilized forceps the antifungal discs were applied onto the surface of the inoculated plate. The plates were incubated at 35ºC for 48 hours. The plates were read at 24hrs and 48hrs.[51]

The following commercial Hi-Media antifungal discs were used

Amphotericin B 100 units

Itraconazole 10µg

Fluconazole 10µg

Interpretation

Zone diameters were measured to the nearest whole millimeter at the point where there was prominent reduction of growth.[89]

Broth microdilution method

This method was used for filamentous fungi

The Clinical and Laboratory Standards Institute (CLSI) subcommittee on Antifungal Susceptibility Tests has developed a reproducible procedure for antifungal susceptibility testing of filamentous fungi by a broth

microdilution format the M-38A document. Recently, an agar diffusion method has been developed for testing filamentous fungi by disc diffusion methodology.

Inoculum preparation

Mould stock inoculum suspensions were prepared from fresh mature (7 day old) cultures grown on Potato dextrose agar following CLSI guidelines. A conidial suspension was prepared by flooding each slant with sterile distilled water. To reduce the hydrophobicity of the conidia and to aid with the formation of uniform conidial suspension of *Aspergillus spp.*, Tween 80 was added to the sterile distilled water.

The resulting suspension was permitted to stand for 5 minutes to allow large particles to settle down. The suspension was then adjusted spectrophotometrically at 530 nm to the optical density range of 0.09-0.11 for *Aspergillus spp.* to get an inoculum size of 1.6×10^6 CFU/ ml.[89]

Procedure

This was done as per CLSI document M 27-A 2 for Yeasts, CLSI, Pennsylvania, USA 2002.16

The test was performed in a 96 well microtitre plate using standard RPMI1640 medium. MIC range of Amphotericin B : 0.03 – 16 µg/ml

The following standard strains were tested each time to ensure quality control:

Aspergillus flavus ATCC 204304

Aspergillus fumigatus ATCC 204305

Due to the lack of defined breakpoints for Amphotericin B, isolates showing an MIC of 1.0 µg/ ml were taken as susceptible and those with MIC >1 µg /ml were considered as resistant.[102] The MIC for Amphotericin B was defined as the lowest concentration in which an optically clear well was observed.[32]

RESULTS

TABLE: 1

SEX DISTRIBUTION OF CASES (n = 100)

Total number of cases	Male	Female
100	67 (67%)	33 (33%)

$P < 0.05$

67% of the cases with lower respiratory tract involvement were males and 88% were females. This is statistically significant.

TABLE: 2

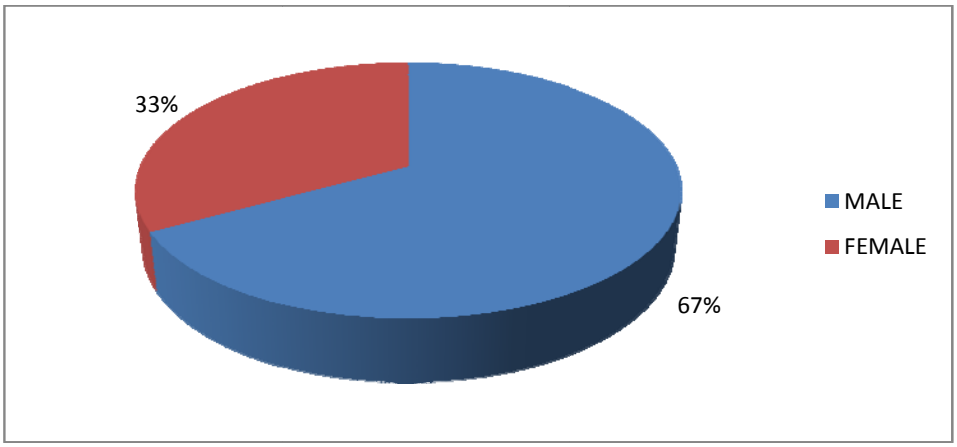
AGE DISTRIBUTION OF CASES (n = 100)

Age group	No. of cases
< 20 years	7 (7%)
21 – 40 years	23 (23%)
41 – 60 years	52 (52%)
> 61 years	18 (18%)

$P < 0.001$

52% of cases were in the 41-60 years age group and 23% of cases in the 21-40 years of age. This is statistically significant.

SEX DISTRIBUTION OF THE CASES



AGE DISTRIBUTION OF THE CASES

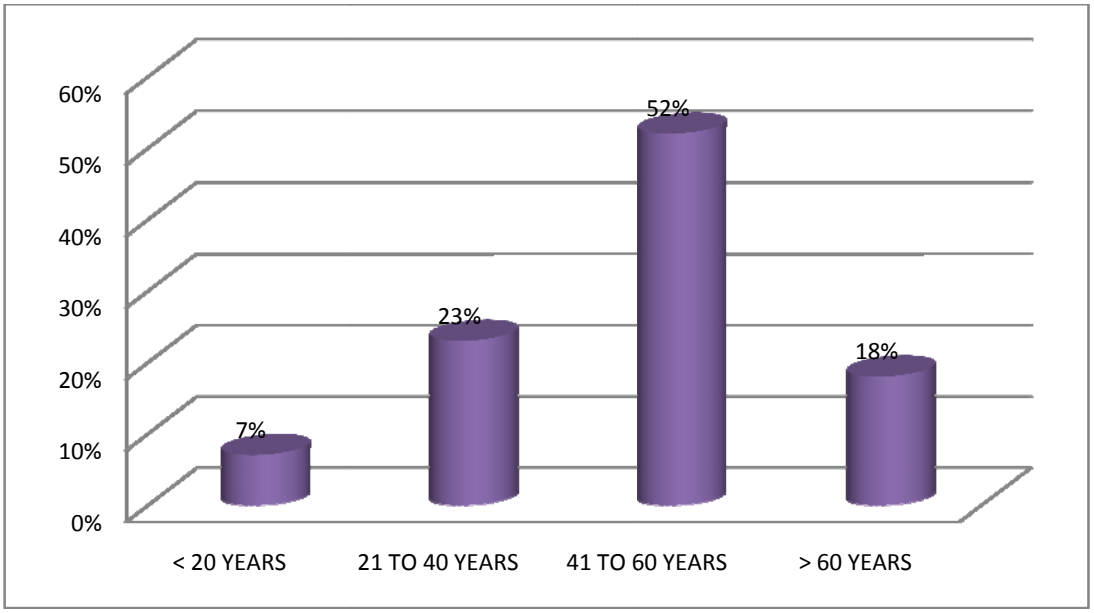


TABLE: 3

CASE DISTRIBUTION

Pneumonia	33 (33%)
Lung abscess	16 (16%)
Lung tumours	15 (15%)
Pulmonary tuberculosis	12 (12%)
Transplant recipients with pulmonary infiltration	6 (6%)
Hydropneumothorax	4 (4%)
Chronic bronchitis	4 (4%)
Renal failure with LRTI	2 (2%)
Bronchoalveolar carcinoma	2 (2%)
Allergic broncho pulmonary aspergillosis	2 (2%)
Aspergilloma	1 (1%)
Carcinoma parotid with secondaries neck	1 (1%)
Mediastinal fibrosis	1 (1%)
Sarcoidosis	1 (1%)

Pneumonia (33%) was the most common LRTI evaluated by bronchoalveolar lavage sampling, followed by lung abscess (16%) and tumours (15%).

TABLE: 4

CULTURE POSITIVITY OF BRONCHOALVEOLAR LAVAGE SAMPLES (n=100)

Total no. of samples	No. of culture positive samples	Percentage of positivity	Total no. of Bacteria & Fungi isolated
100	60	60	69

Out of the total 100 samples, 69 aetiological agents were isolated from 60 samples with culture positivity of 60%

TABLE: 5

ORGANISMS ISOLATED FROM BRONCHOALVEOLAR LAVAGE

(n=69)

Gram positive cocci	7	10.14%
Gram negative bacilli	47	68.1%
Fungal isolates	15	21.7%

$P < 0.0001$

Majority of the isolated agents were gram negative bacilli (68%) followed by fungal isolates (21.7%). This is statistically significant.

TABLE: 6

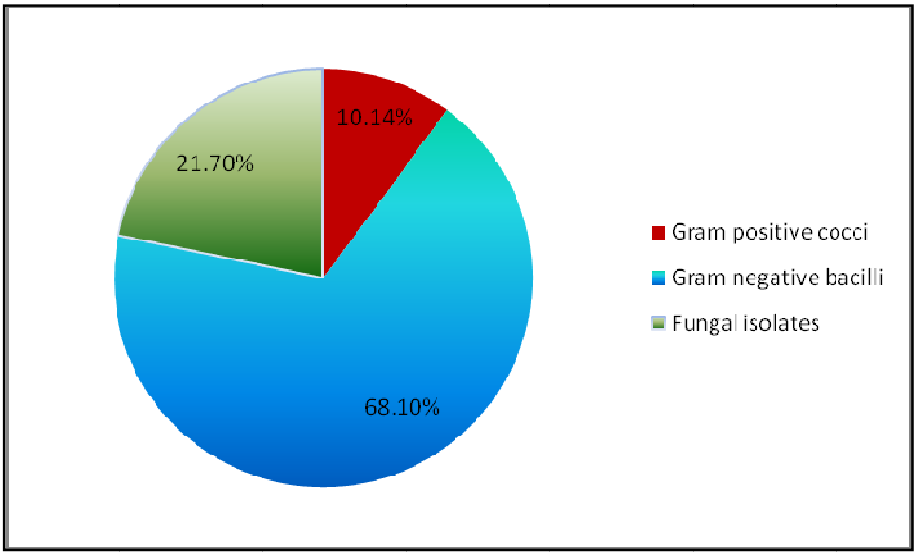
BACTERIAL ISOLATES IN BRONCHOALVEOLAR LAVAGE

(n=54)

Klebsiella pneumonia	12	22.2%
Klebsiella oxytoca	6	11.1%
Pseudomonas aeruginosa	9	16.7%
Acinetobacter spp.	9	16.7%
Staphylococcus aureus	6	11.1%
Escherichia coli	5	9.3%
Proteus vulgaris	4	7.4%
Streptococcus pneumonia	1	1.9%
Proteus mirabilis	1	1.9%
Citrobacter koseri	1	1.9%

Klebsiella spp. were the most common bacterial isolates in broncho alveolar lavage accounting for 33.3% of infection followed by *Pseudomonas aeruginosa* and *Acinetobacter spp.* (16.7%).

ORGANISMS ISOLATED



BACTERIAL ISOLATES

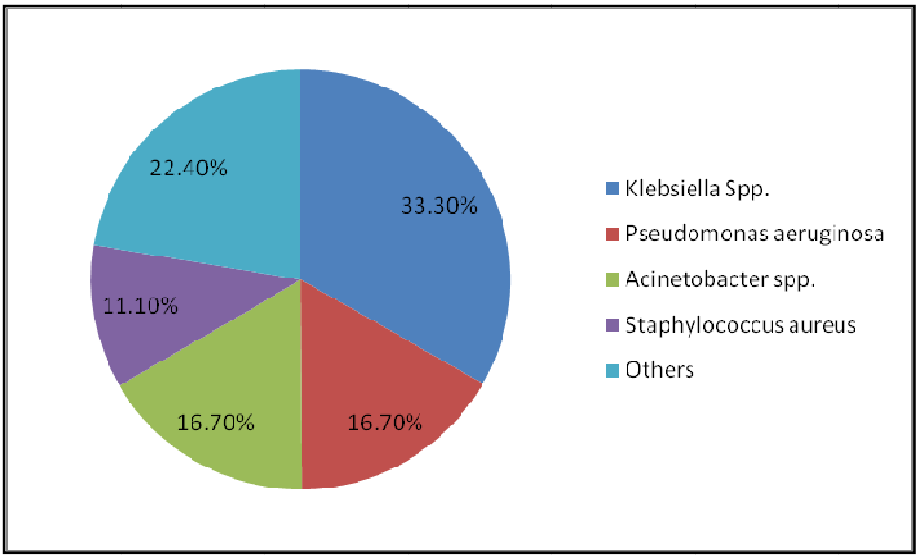


TABLE: 7

FUNGAL ISOLATES IN BRONCHOALVEOLAR LAVAGE (n=15)

Candida spp.	11	73.3%
Aspergillus flavus	2	13.3%
Aspergillus fumigatus	1	6.7%
Syncephalastrum spp.	1	6.7%

Among the fungal isolates, *Candida spp.* were the most common isolates (73.3%), followed by *Aspergillus spp.* (20%).

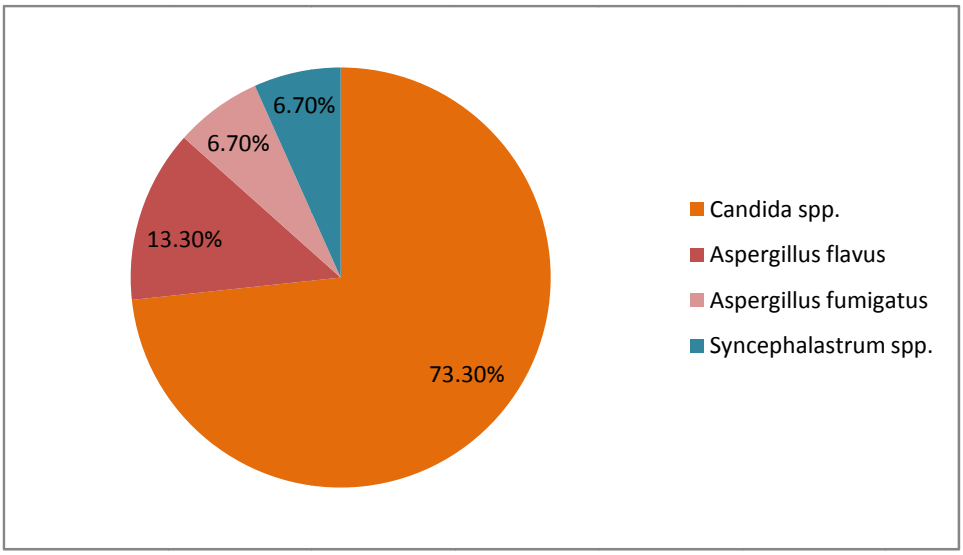
TABLE: 8

NATURE OF THE ISOLATES (n=60)

Monomicrobial	
Bacterial	39 (65%)
Fungal	12 (20%)
Polymicrobial	
Bacterial with bacterial	6 (10%)
Bacterial with fungal	3 (5%)

Of the culture positive samples, 15% showed mixed infections. Among them 10% was bacterial with bacterial isolate and 5% bacterial with fungal. Polymicrobial infections were more common in Pneumonia and lung abscess.

FUNGAL ISOLATES



AFB POSITIVITY

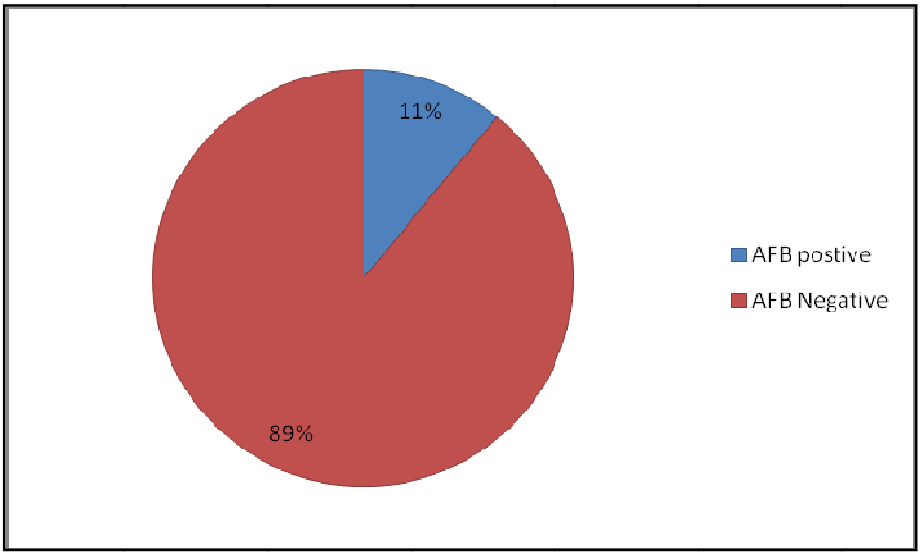


TABLE: 9

SMEAR POSITIVITY FOR ACID FAST BACILLI BY ZIEHL NEELSEN METHOD

Total No. of samples	100
Positive by AFB stain	11 (11%)
Negative by AFB stain	89 (89%)

Acid Fast Bacilli were found in 11% of samples.

TABLE: 10

ANTIBACTERIAL SENSITIVITY PATTERN OF GRAM-POSITIVE ORGANISMS BY KIRBY BAUER'S METHOD (n=7)

Organism	Penicillin	Ampicillin	Oxacillin	Erythromycin	Cotrimoxazole	Cefotaxime	Amikacin	Cipro floxacin	Vanco mycin
Strep. pneumoniae (n=1)	1(100%)	1(100%)	1(100%)	1(100%)	1(100%)	1(100%)	1(100%)	1(100%)	1(100%)
Staph.aureus (n=6)									
MSSA(2)	2(100%)	2(100%)	2(100%)	2(100%)	2(100%)	2(100%)	2(100%)	2(100%)	2(100%)
MRSA(4)	0	0	0	3(75%)	3(75%)	0	4(100%)	3(75%)	4(100%)

MSSA – Methicillin Sensitive Staphylococcus Aureus

MRSA – Methicillin Resistant Staphylococcus Aureus

Among the *Staphylococcus aureus* isolates, 66.7% was methicillin resistant. All the *Staphylococcus aureus* were sensitive to vancomycin.

RESISTANCE PATTERN IN STAPHYLOCOCCUS AUREUS

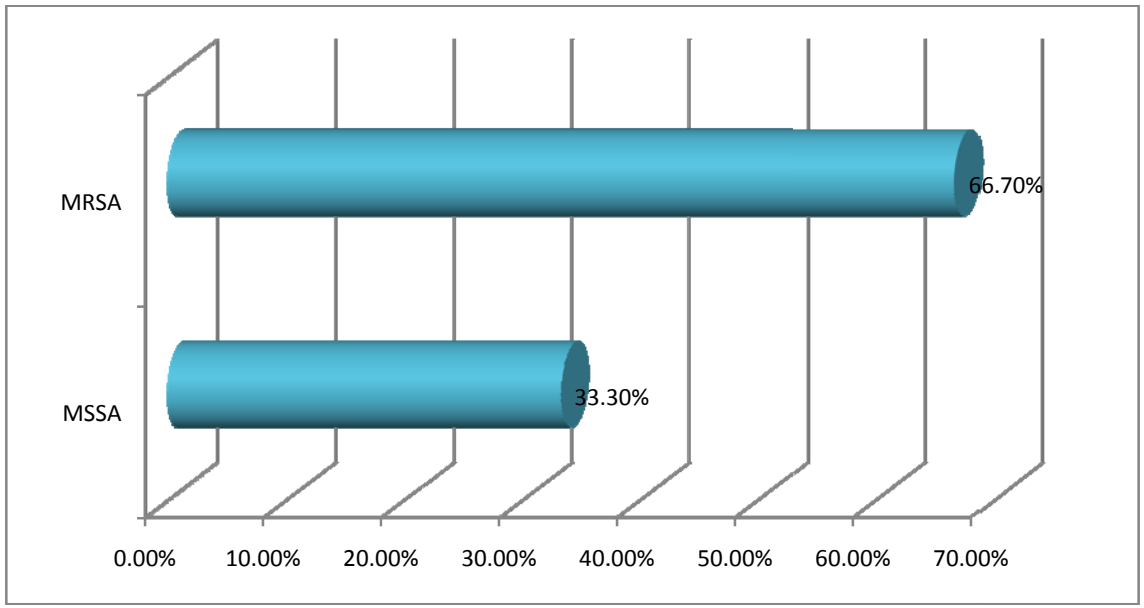

MRSA
MSSA
66.70%
33.30%
0.00%
10.00%
20.00%
30.00%
40.00%
50.00%
60.00%
70.00%

TABLE:11

ANTIBACTERIAL SENSITIVITY PATTERN OF GRAM-NEGATIVE ORGANISMS BY KIRBY BAUER'S METHOD (N=47)

Organism	Ampicillin	Cotrimoxazole	Cefotaxime	Amikacin	Ceftazidime	Gentamicin	Ciprofloxacin	Imipenem
Klebsiella pneumoniae (n=12)	3(25%)	6(50%)	7(58.3%)	10(83.3%)	8(66.7%)	9(75%)	11(91.6%)	12(100%)
Klebsiella oxytoca (n=6)	3(50%)	2(33.3%)	3(50%)	5(83.3%)	4(66.7%)	3(50%)	4(66.7%)	6(100%)
Pseudomonas aeruginosa (n=9)	2(22.2%)	3(33.3%)	5(55.6%)	8(88.9%)	7(77.8%)	6(66.7%)	8(88.9%)	9(100%)
Acinetobacter spp. (n=9)	6(66.7%)	8(88.9%)	6(66.7%)	9(100%)	8(88.9%)	8(88.9%)	8(88.9%)	9(100%)
Escherichia coli (n=5)	3(60%)	4(80%)	4(80%)	5(100%)	5(100%)	5(100%)	3(60%)	5(100%)
Proteus vulgaris (n=6)	4(66.7%)	5(83.3%)	5(83.3%)	6(100%)	5(83.3%)	4(66.7%)	4(66.7%)	6(100%)
Proteus mirabilis (n=1)	1(100%)	0	1(100%)	1(100%)	1(100%)	0	0	1(100%)
Citrobacter koseri (n=1)	1(100%)	1(100%)	1(100%)	1(100%)	1(100%)	0	0	1(100%)

All the Gram Negative Isolates were sensitive to Imipenem.

ANTIBIOTIC SENSITIVITY PATTERN OF KLEBSIELLA PNEUMONIAE

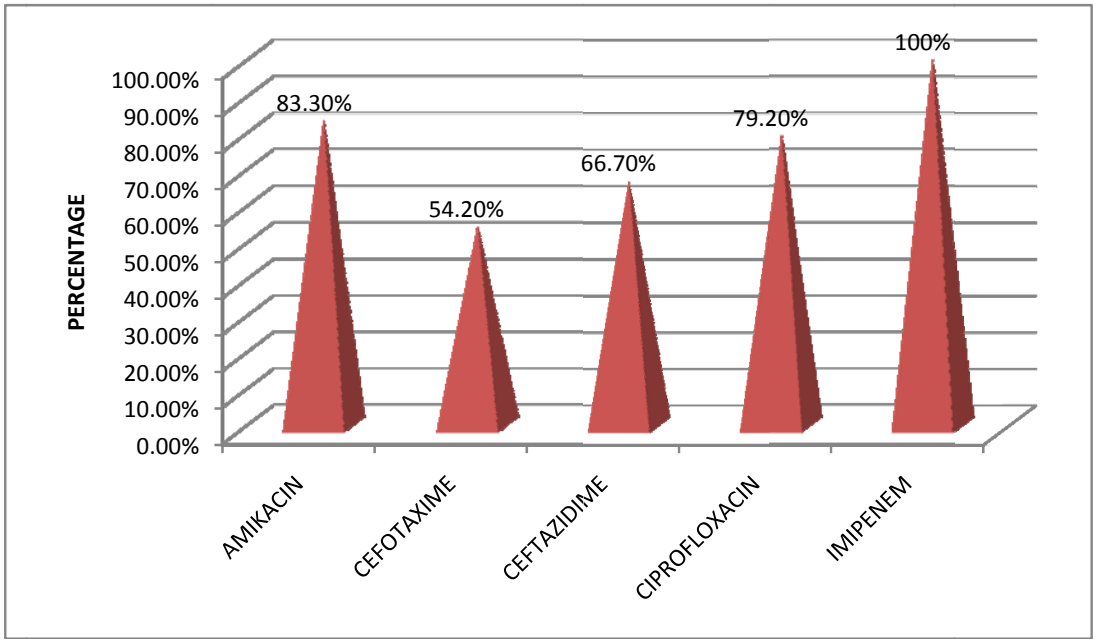


TABLE: 12

DISTRIBUTION OF RESULTS OF SCREENING TEST FOR ESBL

(Cefotaxime: zone of inhibition > 27 mm – sensitive, < 27 mm – resistant)

Organism	**Sensitive to 3rd gen Cephalosporins**		**Resistant to 3rd gen Cephalosporins**	
	No. of isolates	**Percentage**	**No. of isolates**	**Percentage**
Klebsiella pneumoniae (n=12)	7	58%	5	42%
Klebsiella oxytoca (n=6)	3	50%	3	50%
Escherichia coli (n=5)	4	80%	1	20%
Proteus vulgaris (n=4)	3	75%	1	25%

Eight isolates of *Klebsiella spp*., one of *Escherichia coli* and one of *Proteus vulgaris* were resistant to third generation cephalosporins.

TABLE: 13

COMPARISON OF SCREENING TESTS WITH DDST AND PCDDT

Organism	**No. of screened isolates for ESBL**	**DDST**	**PCDDT**
Klebsiella pneumoniae (n=12)	5 (42%)	4 (33.3%)	4 (33.3%)
Klebsiella oxytoca (n=6)	3 (50%)	2 (33.3%)	2 (33.3%)
Escherichia coli (n=5)	1 (20%)	1 (20%)	1 (20%)
Proteus vulgaris (n=4)	1 (25%)	1 (25%)	1 (25%)

10 isolates were selected and subjected for confirmatory tests. 8 isolates were confirmed as ESBL Producers by PCDDT.

TABLE: 14

ANTIFUNGAL SUSCEPTIBILITY OF CANDIDA SPP. BY DISC DIFFUSION METHOD (n=11)

Isolate	Amphotericin B		Fluconazole		Itraconazole	
	Sensitive	**Resistant**	**sensitive**	**resistant**	**sensitive**	**Resistant**
Candida albicans (n=8)	8(100%)	-	8(100%)	-	8(100%)	-
Candida tropicalis (n=3)	3(100%)	-	1(33.3%)	2(66.7%)	3(100%)	-

All the isolates of *Candida albicans* and *Candida tropicalis* were sensitive to Amphotericin B and Itraconazole. 2 isolates (66.7%) of *Candida tropicalis* showed resistance to Fluconazole.

TABLE: 15

ANTIFUNGAL SUSCEPTIBILITY OF ASPERGILLUS BY MICROBROTH DILUTION METHOD

Isolates	Amphotericin B	
	Sensitive **MIC ≤ 1 µg/ml**	**Resistant** **MIC ≥ 1 µg/ml**
Aspergillus fumigatus (n=1)	1(100%)	0
Aspergillus flavus (n=2)	2(100%)	0

All the *Aspergillus spp.* were susceptible to Amphotericin B.

TABLE: 16

SCREENING BY DIRECT GRAM'S STAIN

Gram's Stain	Bacterial Culture		Total
	Positive	Negative	
Positive	43	2	45
Negative	11	44	55
Total	54	46	100

Gram's staining showed sensitivity of 79.6% and specificity of 95.6%.

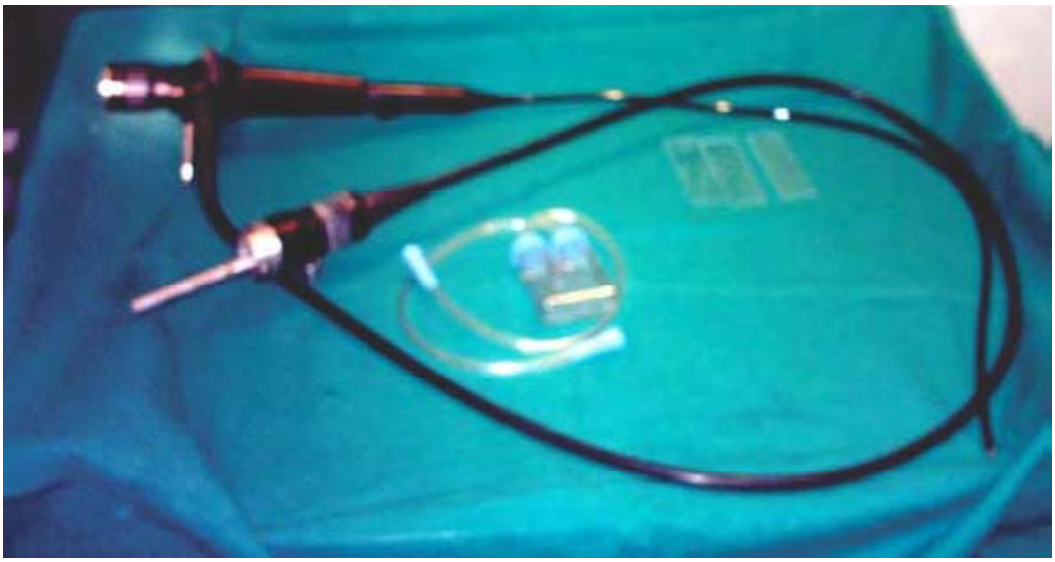
TABLE: 17

SCREENING BY 10% KOH MOUNT FOR FUNGAL ELEMENTS

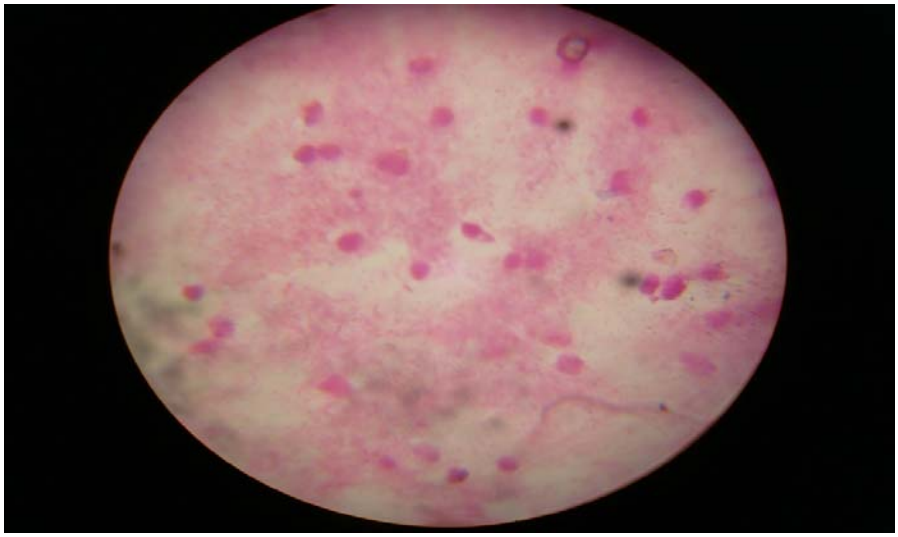
10% KOH Mount	Fungal Culture		Total
	Positive	Negative	
Positive	14	-	14
Negative	1	85	56
Total	15	85	100

10% KOH mount screened for fungal elements showed sensitivity of 93.3%.

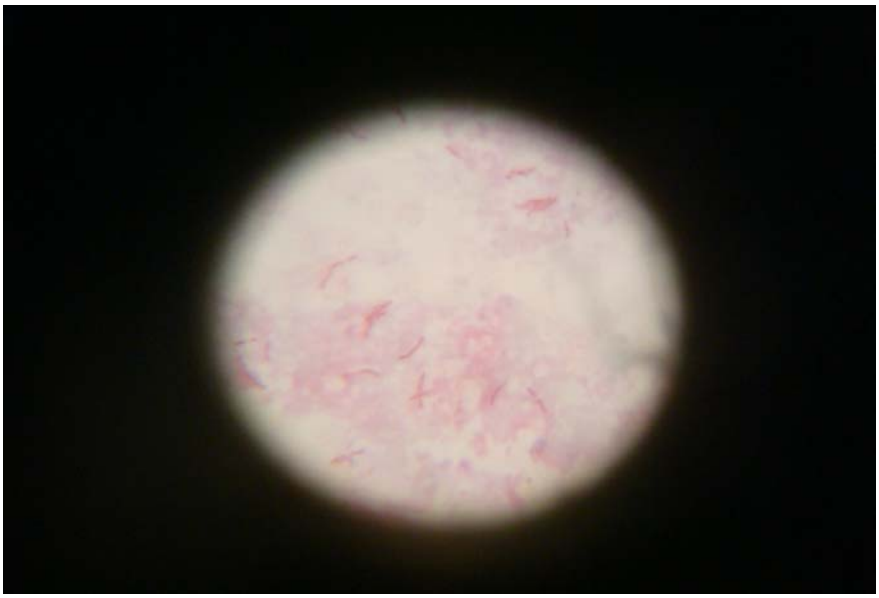
FIBRE OPTIC BRONCHOSCOPE



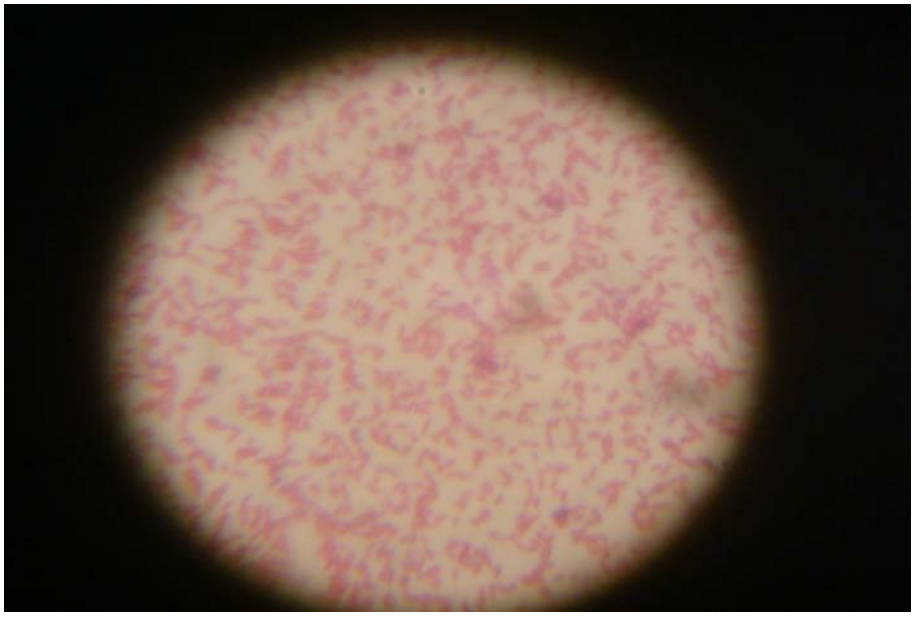
DIRECT GRAM STAIN



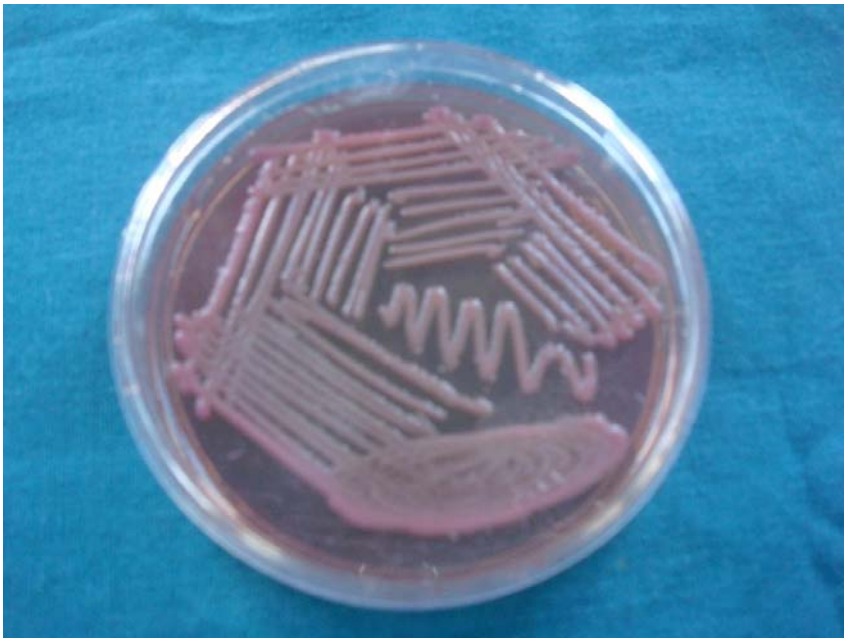
ACID FAST BACILLI



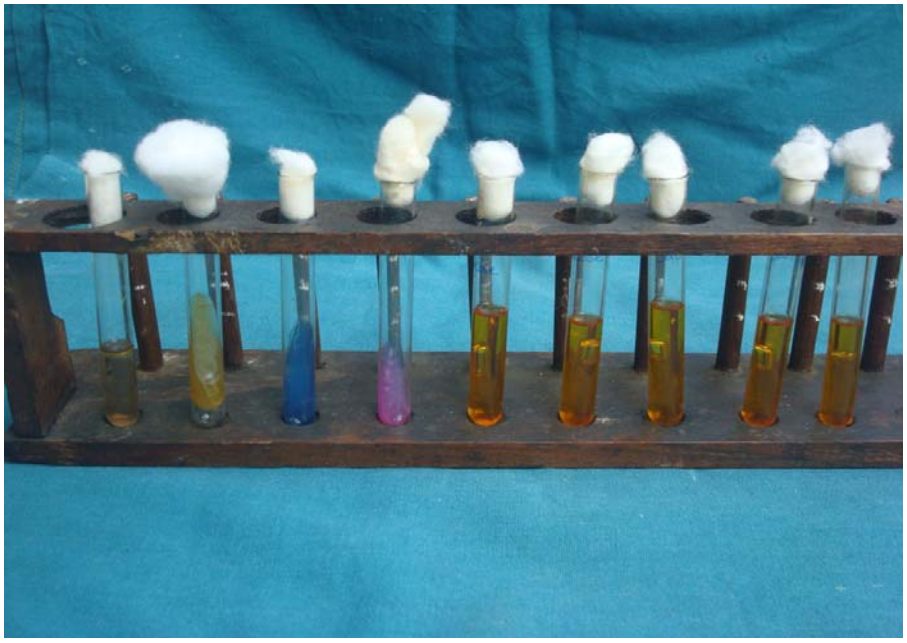
GRAM NEGATIVE BACILLI



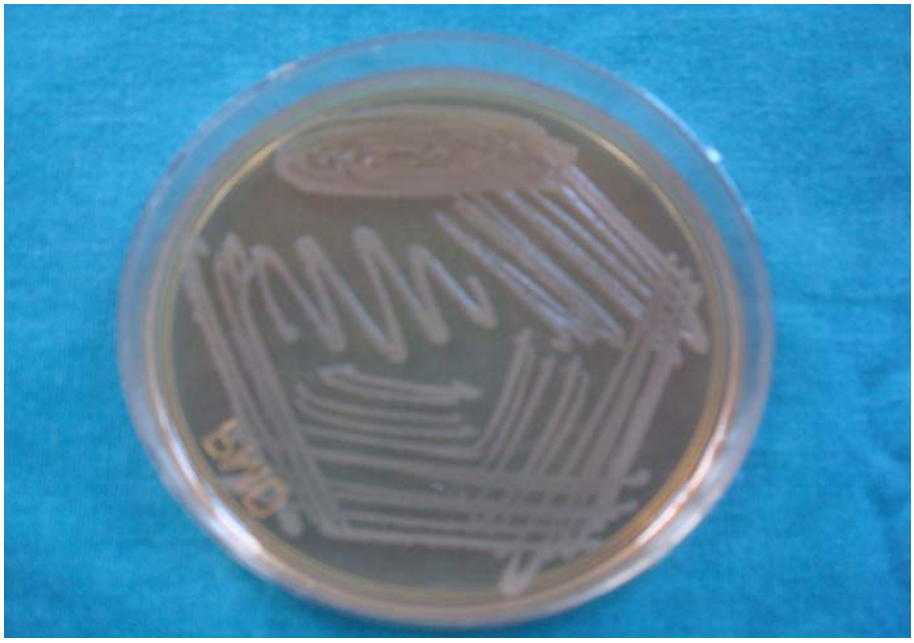
GROWTH OF KLEBSIELLA PNEUMONIAE IN MACCONKEY AGAR



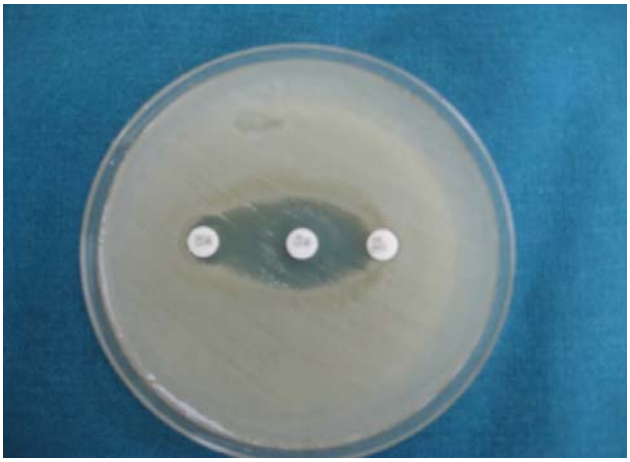
BIOCHEMICAL REACTION OF KLEBSIELLA PNEUMONIAE



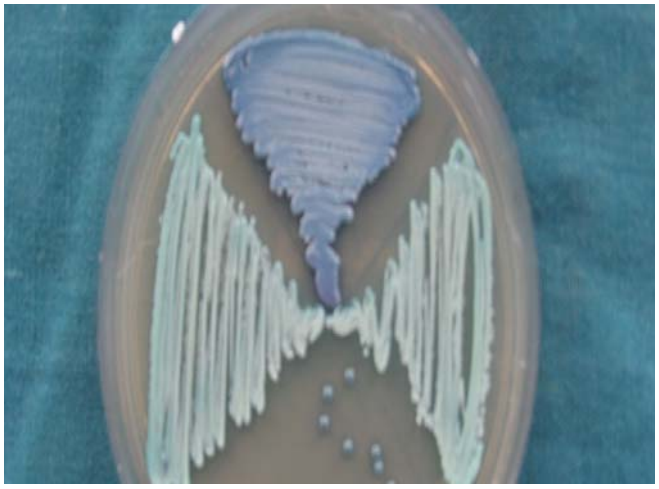
GROWTH OF PSEUDOMONAS SPP IN MACCONKEY AGAR



DISTORTION OF ZONE OF INHIBITION FOR DETECTION OF ESBL PRODUCERS

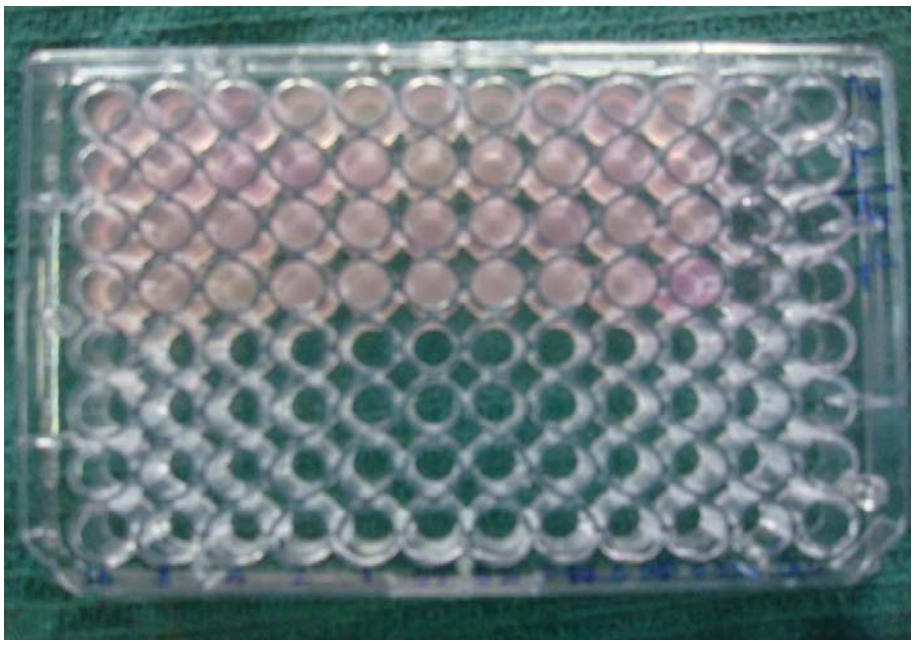


SPECIATION OF CANDIDA SPP USING CHROM AGAR



ANTIFUNGAL SUSCEPTIBILITY OF CANDIDA SPP BY DISC DIFFUSION METHOD

ANTIFUNGAL SUSCEPTIBILITY TESTING FOR AMPHOTERICIN B USING MICROBROTH DILUTION METHOD



DISCUSSION

Lower respiratory tract infections are the major cause of morbidity and mortality worldwide. They remain the leading cause of deaths among the infectious diseases.[105] Hence earlier diagnosis and treatment is essential.

The etiological agents of lower respiratory tract infection are not determined in 50% of patients despite extensive diagnostic testing .[6]

Most of the specimens used for the diagnosis of pulmonary infections have drawbacks like contamination with oropharyngeal flora or invasive nature of some of the procedures.

Bronchoalveolar lavage is a deeper sampling technique and is now reported to have value in diagnosing pulmonary infections.[25] Bronchoalveolar lavage is especially suitable for detecting cysts of *Pneumocystis jirovecii*, fungal elements and *Mycobacteria*[23] particularly in immuncompromised patients.

Various workers stressed upon the utility of bronchoalveolar lavage in the diagnosis and monitoring of lung diseases.[18] This study was aimed to evaluate the common aerobic bacterial and fungal isolates in bronchoalveolar lavage and to analyse the antimicrobial sensitivity pattern of the isolated organisms.

In this study the occurrence of lower respiratory tract pathology was found to be more common in males (67%) than females (33%). $P < 0.05$

significant. [Table:1] This is similar to the study by Karen C.Caroll et al (2002), which revealed 62% cases in males.[75] Similar findings were observed in the study by Reimer et al (1998) and Leatherman et al with 65% and 70% of predominance in males respectively.[101,81]

The distribution of cases was found to be more common in the age group of 41-60 years (52%) in this study. $P < 0.001$ significant. [Table:2] This observation correlates with the study by Karen et al (2002), who reported higher prevalence among patients more than 40 years of age.[75] The study by Grayston et al (1994), also showed a higher prevalence in 41-60 years age group.[59]

The most common LRTI that required bronchoalveolar lavage analysis in this study was found to be pneumonia (33%) followed by lung abscess and tumours [Table:3]. This was similar to the study by Reimer et al (1998) and Kahn et al, which showed higher prevalence of pneumonia among the lower respiratory infections.[101,73]

According to this study, the etiological agents were isolated in 60 Bronchoalveolar lavage samples (60%). Of these 60 positive samples, 39 (65%) had single bacterial isolate, 12 (20%) had single fungal isolate and 9 (15%) had mixed infections. Of the mixed infections, 10% of the sample showed more than one bacterial isolate and 5% showed bacterial with fungal growth. [Table:8]

These observations were similar to the study be G.C. Bhatia et al (2006), which revealed pure bacterial growth in 61% and pure fungal growth in 16.7% in bronchoalveolar lavage.[18] This result also correlates well with the study by Karen et al (2002), in which the mixed infections were about 18%.

Among the 69 isolates, 54 isolates (78.3%) were bacterial and 15 isolates (21.7%) were fungal. $P < 0.05$ significant.

In the present study, aerobic gram negative bacilli were found in 68.1% of cases. $P < 0.0001$ significant. [Table:5]. The predominant isolate was *Klebsiella spp.(Klebsiella pneumoniae and Klebsiella oxytoca*) (33.3%) followed by *Pseudomonas aeruginosa* (16.7%) and *Acinetobacter spp.* (16.7%). Other gram negative bacilli were *Escherichia coli* (9.3%), *Proteus spp.* (9.3%) and one isolate of *Citrobacter koseri*(1.9%). Gram positive cocci were found in 12.9% of cases. *Staphylococcus aureus* was the predominant isolate [Table:6]

A study conducted by Crystal et al (1996) showed the predominant isolates in bronchoalveolar lavage were gram negative bacilli particularly in hospital acquired infections.[37] Among those, *Klebsiella pneumoniae* was found in 30%. These observations were similar to the study by Bhatia et al (2006), where the percentage of *Klebsiella spp.* was 32%.[18]

The next common isolates following *Klebsiella spp.* were *Pseudomonas aeruginosa* (16.7%) and *Acinetobacter spp.* (16.7%). This correlates well with the study by Frederick et al (1998).[56]

In this study among the culture positive cases, 22% were positive for fungal isolates. Among the fungal isolates, 73.3% was *Candida spp.* and 20% *Aspergillus spp (Aspergillus flavus and Aspergillus fumigatus).* with *Candida albicans* being the most common isolate [Table:7]

This is similar to the study by Bjermer et al (1992), where the fungal isolates in bronchoalveolar lavage was 20% and *Candida albicans* was the most common isolate.[19] This also correlates with the study by Bhatia et al (2006), in which *Candida albicans* was the most common fungal isolate forming 12% of the total culture positive samples and 83.3% of the total fungal isolates.[18]

In the present study, all the samples of Bronchoalveolar lavage fluids were screened for *Mycobacterium tuberculosis* by Ziehl Neelsen staining method. Among them, 11 samples (11%) were found to be positive for Acid Fast Bacilli [Table:9]

In a study by Purohit et al (2000), an early diagnosis of pulmonary tuberculosis was made in 13% by positive microscopy for AFB on BAL.[99] In another study by Getachew et al *Mycobacterium tuberculosis* was isolated in 19% sample of BAL.[57]

The Antibiogram performed for all the bacterial isolates in this study by Kirby-Bauer Disc Diffusion Method showed the *Klebsiella spp.* with 83.3% sensitivity to Amikacin, 54.2% sensitivity to Cefotaxime, 66.7% to Ceftazidime, 79.2% to Ciprofloxacin and 100% to Imipenem [Table:11]

The second most common bacilli *Pseudomonas aeruginosa* showed 88.9% sensitivity to Amikacin, 55.6% to Cefotaxime and 100% to Imipenem. *Acinetobater spp.* showed 100% sensitivity to Amikacin and Imipenem.

All the isolates were 100% sensitive to Imipenem. The next most effective drug was found to be Amikacin with 94% sensitivity.

These observations were similar to the studies of Bartlett JG et al (2002) and Sharma et al (2006), where Imipenem showed 100% sensitivity and Amikacin was effective against 93 to 95% isolates.[8,109]

Out of the 6 strains of *Staphylococcus aureus*, 2 strains (33.3%) showed sensitivity to Oxacillin (1µg disc) by disc diffusion method; *Methicillin sensitive Staphylococcus aureus* (MSSA) strains were also sensitive to Penicillin (100%), Ampicillin (100%), Cotrimoxazole (100%), Ciprofloxacin (100%), Amikacin (100%) and Erythromycin (100%) [Table:10]

The other 4 strains (66.7%) of *Staphylococcus aureus* showed resistance to Oxacillin (1µg disc) (MRSA). All the 6 isolates of *Staphylococcus aureus* were sensitive to Vancomycin (100%).

Among the gram negative bacilli, 42% of *Klebsiella pneumoniae*, 50% of *Klebsiella oxytoca*, 20% of *Escherichia coli* and 25% of *Proteus vulgaris* were found to be Extended Spectrum β Lactamase producers by screening method [Table:12]

By Phenotypic Confirmatory Disc Diffusion Test, 33.3% of *Klebsiella pneumoniae*, 33.3% of *Klebsiella oxytoca*, 20% of *Escherichia coli* and 25% of *Proteus vulgaris* were confirmed as ESBL producers [Table:13]

Jarlier et al reported that *Klebsiella pneumoniae* (48%) was the most frequent ESBL producing organism followed by *Escherichia coli* (16.8%) in Bronchoalveolar lavage which correlates well with our study.[71] In another study by Baker et al 2006, *Klebsiella pneumoniae* was found to be the most common ESBL producing organism.

Among the 11 *Candida spp.* screened by Disc Diffusion Method in this study, all the isolates of both *Candida albicans* and *Candida tropicalis* showed 100% sensitivity to Amphotericin B and Itraconazole. Fluconazole resistance was observed in 2 isolates (66.7%) of *Candida tropicalis*. [Table:14]

A study by Law D et al reported that 48% of *Candida tropicalis* in his study was resistant to fluconazole.[80] Rex et al (1996), reported that all the isolates of *Candida albicans* in his study were sensitive to Amphotericin B and observed 54% resistance to Fluconazole for *Candida tropicalis.*[102]

In the present study Antifungal susceptibility was performed on the 3 isolates of *Aspergillus spp.* for Amphotericin B by Broth microdilution method. It was observed that all the 3 isolates (100%) of *Aspergillus spp.* were sensitive to Amphotericin B with MIC ≤ 1 µg/ml [Table:15]

This result correlates well with the study by Espinel-Ingroff et al (2007), where the sensitivity of *Aspergillus spp.* to Amphotericin B was found to be 100%.[52] In a study by Bez et al 2002, *Aspergillus flavus* showed 100% susceptibility to Amphotericin B.[16]

In evaluating the screening tests for rapid diagnosis of the bacterial and fungal isolates in bronchoalveolar lavage, Gram's stain examination and 10% Potassium hydroxide (KOH) mount of the BAL fluid were analysed.

In the present study, the Gram's stain showed sensitivity of 79.6% and specificity of 95.6% (Table:16). Prekates et al (1998), reported 77% sensitivity and 87% specificity of Gram's stain examination of Bronchoalveolar lavage.[98] In a study by Allaouchiche et al (2007), the sensitivity of Gram's stain was 90.2% and specificity was 73.7%.[2]

In this study 10% KOH mount examination for fungal elements showed sensitivity of 93.3% (Table-17). This correlates with the study of Vajpayee.R.B, et al, (1993), which revealed 94.3% sensitivity of 10% KOH mount examination. Bharathi M J et al (2007) reported 99% sensitivity for 10% KOH mount.[118,17]

The results of the present study showed the vital role of Gram's stain examination and 10% KOH mount in the diagnosis of Lower respiratory tract infections. Although culturing of microbial pathogens is considered to be the gold standard, direct microscopic evaluation of smears provide immediate information about the aetiological agents and aid in early initiation of antimicrobial therapy.

SUMMARY

- Totally 100 samples of Bronchoalveolar fluid were analysed in detail. Bacterial and fungal isolates were found in 60 (60%) samples.
- Male preponderance of lower respiratory tract infections was observed in this study.
- The age group most commonly affected was between 41-60 years.
- Pneumonia was found to be the most common lower respiratory tract infection.
- Majority of the isolates were bacterial (78.3%) with gram negative bacilli (68.1%) being the most common.
- Among the gram negative bacilli, *Klebsiella spp.* (Klebsiella pneumonia and Klebsiella oxytoca) (33.3%) was the most common followed by *Pseudomonas aeruginosa* and *Acinetobacter baumanii* (16.7% each)
- Fungal isolates were less common (21.7%). The predominant fungal isolate was *Candida albicans* followed by *Aspergillus spp.*
- Acid Fast Bacilli were found in 11% of the total samples.
- All the bacterial isolates were sensitivie to Imipenem and majority of the isolates were sensitive to Amikacin.
- Among the *Staphylococcus aureus*, 33.3% was methicillin sensitive and 66.7% was methicillin resistant.

- ❖ All the 4 MRSA strains were sensitive to Vancomycin.
- ❖ The incidence of ESBL producing *Klebsiella spp.* was 33.3% and *Escherichia coli* was 20%.
- ❖ All the isolates of *Candida spp.* were sensitive to Amphotericin B and Itraconazole. 66.7% of *Candida tropicalis* showed resistance to Flucanazole by Disc Diffusion method.
- ❖ All the isolates of *Aspergillus spp.* were sensitive to Amphotericin B by Broth Microdilution method.
- ❖ Gram's stain and 10% KOH mount procedures were found to be highly sensitive as rapid screening tests for isolating the bacterial and fungal isolates in Bronchoalveolar lavage.

CONCLUSION

Lower respiratory tract infections are the major cause of morbidity and mortality worldwide. But the etiological agents were not determined in 50% of cases despite extensive diagnostic testings. Nowadays, analysis of Bronchoalveolar lavage plays a definite role in diagnosing pulmonary infections. On analysing the BAL fluid, *Klebsiella spp.* and *Candida spp.* were the most common bacterial and fungal isolates respectively. From the present study, the vital role of microbiological analysis of BAL fluid is clearly evident since the clinical features alone are not adequate to confirm infections. A simple Gram's stain and KOH preparation were highly beneficial as rapid screening tests for diagnosis. Antimicrobial susceptibility testing was done for the bacterial and fungal isolates. Precise identification of the causative organisms and timely institution of appropriate antimicrobial therapy based on the prevailing sensitivity pattern of the bacterial and fungal isolates could reduce the morbidity and mortality of lower respiratory tract infections.

PROFORMA

S.No. : Date: I.P./O.P.No.:

Name : Age/Sex: Occupation:

Address :

Presenting Complaints: Duration:

Number of days hospitalized:

Provisional Diagnosis:

Date of bronchoscopy:

H/O present illness:

- Fever
- Cough with expectoration
- Breathlessness
- Weight loss
- Haemoptysis

H/O past illness:

- Similar illness before
- Tuberculosis
- Diabetes
- Hypertension

- Smoking
- Alcohol intake
- Blood transfusion

Family history:

H/O contact with known case of tuberculosis

Laboratory evaluation:

- X ray findings
- CT findings
- MRI findings

ABBREVIATIONS

BAL – Bronchoalveolar lavage

MDR – multidrug resistant

CAP – Community acquired pneumonia

HCAP – Health care associated pneumonia

HAP – Hospital acquired pneumonia

VAP – Ventilator associated pneumonia

COPD – Chronic obstructive pulmonary disease

HIV – Human immunodeficiency virus

AIDS – Acquired immunodeficiency syndrome

PSB – Protected specimen brush

LRTI – Lower respiratory tract infection

FOB – Fiberoptic bronchoscopy

PTB – Pulmonary tuberculosis

KOH – Potassium hydroxide

LPCB – Lactophenol cotton blue

CLSI – Clinical and laboratory standards institute

APPENDIX

A. STAINS AND REAGENTS

I. 10% KOH

Potassium hydroxide l0g

Glycerol – l0ml

Distilled water - 80ml

II. Gram staining

- Methyl violet (2%) – l0g Methyl violet in l00ml absolute Alcohol in 1litre of distilled water (Primary stain)
- Grams Iodine – l0g Iodine in 20g KI (fixative)
- Acetone – Decolourising agent
- Carbol fuchsin 1% – Secondary stain

III. Lactophenol cotton blue stain

Lactic acid - 20 ml

Phenol - 20ml

Cotton blue (dye) - 0.5g

Glycerol - 40ml

Distilled water - 20ml.

Culture Media

1. **Mac Conkey agar**

Peptone	-	20g
Sodium taurocholate	-	5g
Distilled Water	-	1 ltr
Agar	-	20g
2% neutral red in 50% ethanol	-	3.5ml
10% lactose solution	-	l00mI

Dissolve peptone and taurocholate in water by heating. Add agar and dissolve it in steamer. Adjust pH to 7.5. Add lactose and neutral red shake well and mix. Heat in free steam (100°C) for 1 hour, then autoclave at 115°C for 15 minutes.

2. **Blood agar (5% sheep blood agar)**

Peptone	-	l0g
Nacl	-	5g
Distilled water	-	1 Ltr
Agar	-	10g

Dissolve ingredients in distilled water by boiling, and add 5% sheep blood (sterile) at 55°C adjust pH to 7.4.

3.Chocolate Agar

Sterile defibrinated blood - 10 ml

Nutrient Agar (melted) - 100 ml

When the temperature was about 75°C, sterile blood was added with constant agitation. After addition of blood, kept in water bath and heating was continued till the blood changed to chocolate colour. Cooled to about 50° C and poured about 15ml into petri dishes with sterile precaution.

4. **Sabouraud's Dextrose Agar With Antibiotics**

Composition of Sabouraud's Dextrose Agar (Emmons Modification)

Dextrose - 20 gm

Peptone - 10 g

Agar - 20 g

Distilled water - 1000 ml

Final pH - 6.9

The ingredients are dissolved by boiling. Gentamycin was dissolved in 10 ml of 95% alcohol and added to boiling medium.

Autoclave at 121°C for 15 minutes, dispense in tubes and allow to cool in slanted position.

5. **Mueller- Hinton Agar**

Beef infusion - 300ml

Caesein hydrolysate	-	17.5g
Starch	-	1.5g
Agar	-	10g
Distilled water	-	1ltr
pH	=	7.4

Sterilise by autoclaving at 1210C for 20 mins

6. RPMI 1640 Broth

Commercially purchased RPMI 1640 media was dissolved in 1000ml of sterile distilled water. The pH was adjusted to 7.0

The medium was sterilized by filtering through a sterile membrane filter with a porosity of 0.22 microns.

7.Cornmeal Agar

Cornmeal	-	40g
Agar	-	15g
Water	-	1 litre

Boil the cornmeal in 1 litre of water for 60 min.Filter through muslin and add the agar. Steam to dissolve, dispense in required amounts and autoclave at 115° C for 30min. allow to cool to 50°C and pour approximately 20 ml amounts into Petri dishes.

8. Sugar Fermentation Medium

Peptone	-	15g
Andrade's indicator	-	10 ml
Sugar to be tested	-	20g
Water	-	1litre

Andrade's indicator is prepared from 0.5% aqueous acid fuchsin to which sufficient 1M sodium hydroxide has been added to turn the colour of the solution yellow.

Dissolve the peptone and Andrade's indicator in 1litre of water and add 20g of the sugar; sugars to be tested generally include glucose ,sucrose, lactose and maltose. Distribute 3ml amounts in standard test tubes containing an inverted Durham tube. Sterilize by steaming at 100 degree C for 30 min on 3 consecutive days.

9. Sugar Assimilation Agar

Basal medium I

Yeast nitrogen base(Difco)

Agar

Water

Steam to dissolve and dispense in 10ml amounts in universal containers. Autoclave at 115 degree C for 15min.

MEDIA REQUIRED FOR BIOCHEMICAL IDENTIFICATION OF BACTERIA

1.Oxidase Reagent

Tetra methyl p-phenelene diamine dihyrochloride-

1% aqueous solution.

2.Catalase

3% hydrogen peroxide

3.Indole test

Kovac's reagent

Amyl or isoamyl alcohol - 150ml

Para dimethyl amino benzaldehyde - 10g

Concentrated hydrochloric acid - 50ml

Dissolve the aldehyde in the alcohol and slowly add the acid. Prepare in small quantities and store in the refrigerator. Shake gently before use.

4.Christensen's Urease test medium

Peptone	-	1g
Sodium chloride	-	5g
Dipotassium hydrogen phosphate	-	2g
Phenol red	-	6ml
Agar	-	20g
Distilled water	-	1ltr

10% sterile solution of glucose - l0ml

Sterile 20% urea solution - l00ml

Sterilize the glucose and urea solutions by filtration. Prepare the basal medium without glucose and urea, adjust to pH 6.8-6.9 and sterilize by autoclaving in a flask at 121°C for 30min. Cool to about 50°C, add the glucose & urea, and tube the medium as slopes.

5.Simmon's Citrate Medium

Koser's medium - 1 ltr

Agar - 20g

Bromothymol blue 0.2% - 40ml

Dispense, autoclave' at 121°C for 15 min and allow to set as slopes

6.Triple Sugar Iron medium

Beef Extract - 3g

Yeast extract - 3g

Peptone - 20g

Glucose - 1g

Lactose - 10g

Sucrose - 10g

Ferric citrate - 0.3g

Sodium chloride - 5g

Sodium thiosulphate - 0.3g

Agar - 12g

Phenol red 0.2% solution - 12ml

Distilled water - 1 ltr

Heat to dissolve the solids, add the indicator solution, mix and tube. Sterilize at 121°C for 15 min and cool to form slopes with deep butts.

7.Glucose phosphate broth

Peptone - 5g

Dipotassium hydrogen phosphate - 5g

Water - 1 ltr

Glucose 10% solution - 50ml

Dissolve the peptone and phosphate and adjust the pH to 7.6. Filter dispense in 5ml amounts and sterilize at 121°c for 15min. Sterilize the glucose solution by filtration and add 0.25ml to each tube.

Methyl Red Reagent

Methyl Red - 10mg

Ethyl alcohol - 30ml

Distilled water - 20ml

Voges Proskauer Reagent

Reagent A: Alpha naphthol - 5g

Ethyl alcohol - 100ml

Reagent B: Potassium hydroxide - 40g

Distilled water - 100ml

8.Peptone water fermentation test medium.

To the basal medium of peptone water, add sterilised sugars of 1% indicator bromothymol blue with Durham's tube.

Basal medium - peptone water

Sugar solutions:

Sugar - 1ml

Dislilled water - l00ml

pH = 7.6.

9.Mannitol motility medium

Agar - 5g

Peptone - 1g

Potassium nitrate - 1g

Mannitol - 2g

Phenol red indicator

Distilled water - l000ml

pH = 7.2

10. Potassium Nitrate Broth

Potassium nitrate (KN03) - 0.2gm

Peptone - 5.0gm

Distilled water - 100ml

The above ingredients were mixed and transferred into tubes in 5 ml amount and autoclaved.

11. Phenyl Alanine Deaminase Test

Yeast Extract 3g

Dl-Phenylalamine 2 g

Disodium hydrogen phosphate lg

Sodium Chloride - 5 g

Agar 12g

Distilled water - 1 lr

pH adjusted to 7.4, distributed in tubes and sterilized by autoclaving at 121° C for 1 5 minutes, allowed to solidify as long slopes.

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